ROLE OF IL-23 INDUCED CYTOKINES IN ORGAN-SPECIFIC AUTOIMMUNITY

Dissertation

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Disclaimer

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- **Haak S*,** Gyülvészi G* and Becher B . Th17 cells in autoimmune disease: changing the verdict. *Immunotherapy* 2009 Mar; 1(2):199-203.

- **Haak S***, Croxford A*, Kreymborg K, Heppner FL, Pouly S, Becher B[#] and Waisman A[#]. IL-17A and IL-17F do not contribute vitally to autoimmune neuro-inflammation. *J Clin Invest.* 2009 Jan; 119(1):61-69

- Kreymborg K, Etzensperger R, Dumoutier L, **Haak S**, Rebollo A, Buch T, Heppner FL, Renauld JC and Becher B. IL-22 is expressed by Th17 cells in an IL-23-dependent fashion, but not required for the development of autoimmune encephalomyelitis. *J Immunol.* 2007 Dec; 179(12):8098-104.

- Gyülvészi G, **Haak S** and Becher B. IL-23-driven encephalo-tropism and Th17 polarization during CNS-inflammation *in vivo*. *Eur J Immunol.* 2009 Jul; 39(7):1864-1869

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Abbreviations

aa	amino acid
AHR	aryl hydrocarbon receptor
AIRE	Autoimmune Regulator
APC	antigen presenting cell
APECED	autoimmune polyendocrinopathy-candidiasis- ectodermal dystrophy
APS	autoimmune polyglandular syndrome
BBB	blood brain barrier
BCB	blood-cerebrospinal barrier
BCR	B cell receptor
CIITA	class II transactivator
CFA	complete Freund's adjuvant
ChIP	chromatin immunoprecipitation
chr.	chromosome
CNS	central nervous system
CRF2	class II cytokine receptor family
CRP	C-reactive protein
CSF	cerebrospinal fluid
CTL	cytotoxic lymphocyte
CTLA	cytotoxic T-lymphocyte-associated protein
DAMP	danger-associated molecular pattern
DC	dendritic cell
DP	double positive
DTH	delayed hypersensitivity
EAE	experimental autoimmune encephalomyelitis
EAU	experimental autoimmune uveoretinitis
EBI3	Epstein Barr virus-induced protein 3
EBV	Epstein–Barr virus
FoxP3	forkhead box P3
γc	common γ-chain
GC	germinal centres
GFAP	glial fibrillary acidic protein
Gfi	growth factor independent
GM-CSF	granulocyte macrophage colony-stimulating factor
HLX	H2.0-like homeobox
IBD	inflammatory bowel disease
icFACS	intracellular cytokine fluorescence activated cell sorting
IFN	interferon
IL	interleukin
IPEX	immune dysregulation, polyendocrinopathy
IRF	interferon regulatory factor
LAP	latency-associated protein
LRR	leucine-rich repeats
LTi	lymphoid tissue inducer cells

MBD	methyl-CpG binding domain protein
MBL	mannose-binding lectin
MBP	myelin basic protein
MHC	major histocompatibility complex
MMP	matrix metalloproteinase
MOG	myelin/ oligodendrocyte glycoprotein
MS	multiple sclerosis
NK cell	natural killer cell
NKT cell	natural killer T cell
NLR	Nod-like receptor
nT_{reg} cell	natural regulatory $CD4^+$ T cell
OD	optical density
PAMP	pathogen-associated molecular pattern
PFA	paraformaldehyde
PGE2	prostaglandin E2
PLP	proteolipid protein
PMN	polymorphonuclear
PRR	pattern recognition receptors
PT	Pertussis toxin
RA	retinoic acid
RANKL	receptor activator of nuclear factor-κB ligand
ROR	retinoid-related orphan receptor
SAS	subarachnoid space
SLT	secondary lymphatic tissue
SOCS	suppressor of cytokine signalling
T-bet	T-box expressed in T cells
TCR	T cell receptor
TF	transcription factor
TGF	transforming growth factor
T_H cell	T helper cell
TIR	Toll-interleukin-1 receptor
TLR	toll-like receptor
TNF	tumour necrosis factor
T_{reg} cell	regulatory T cell
UTR	untranslated region

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Immunity

Immunity is the state of an organism of having sufficient biological defences to avoid, defeat or contain disease. Disease can be caused or facilitated by physical damage (e.g. irradiation, trauma etc.), by infections by pathogens (bacterial, viruses etc.), uncontrolled cell expansion (tumours etc.) and dysregulated immune responses (autoimmunity etc.). The versatility of the immune system must have mainly evolved to fight infection. To do that efficiently the immune response in mammals is divided into a rapid but imprecise and a slower but perfectly matching response.

When the organism is threatened by an invading pathogen innate immune mechanisms provide a body's first line of defence by systemic actions (fever, vaso-dilation etc.), which aid the defence as well as issue repair, and the directed action against stereotypic structures conserved in pathogens. However, systemic alterations like elevation of body temperature and targeted leukocyte action against intruders upon sketchy clues cannot be sustained for a longer period without risk of collateral damage, which is the interference with vital processes or the lasting damage of the organisms own tissues (self). Unmistakable determinants of "pathogenic" are rare and constantly evaded by the pathogen in a process of co-evolution [reviewed in AUGUSTIN R ET AL. 2010]. To ultimately protect against any possible pathogen a plastic, self-adapting immune mechanism has developed that equips the body with lasting protection against specific pathogens (adaptive immunity).

These interlocking systems allow the host to carefully tailor the immune response to the offending pathogen [reviewed in MEDZHITOV R ET AL. 1997]. However, this high degree of flexibility and the licence to reject and eliminate foreign cells and tissues come at a price which becomes visible when immune regulation goes astray or is mislead by the influence of xenobiotics.

Innate mechanisms

The innate arm of the immune system takes part in host defence and tissue repair. In the initiation of an immune response the innate mechanisms are usually the first to act. It includes physical, chemical and cellular barriers. The skin or mucous membranes are physical barriers while chemical barriers include the acidity of the stomach or antimicrobial peptides.

Antimicrobial peptides are produced by a variety of cells, mainly by neutrophils as well as by almost all epithelial cells. They are between six to 60 amino acids (aa) in size, mostly positively charged and often work by disrupting microbial membranes, killing microbes very rapidly. In the context of soluble mediators of innate immunity, also the proteins of the

acute phase response such as the complement system, C-reactive protein (CRP) and mannose-binding lectin (MBL) need to be mentioned.

The components of the complement system are small proteins that are generally synthesized as inactive precursors by hepatocytes. They circulate in the blood and after initial activation through proteases following infection, a highly regulated cascade is initiated that results in (a) lysis of cells, (b) opsonisation promoting phagocytosis, (c) binding to specific complement receptors triggering inflammation and (d) immune clearance removing immune complexes from the circulation. CRP, too, is produced by hepatocytes and binds ligands such as polysaccharide or phosphorylcholine present on the surface of different microbes. Bound CRP promotes phagocytosis and activates the complement system. The latter can also be elicited by MBL, which recognizes mannose-containing molecular patterns present on microbial- but not vertebrate cells.

The cellular part of innate defence involves an array of different cell types such as polymorphonuclear cells (PMN), natural killer (NK) cells, γδT cells and professional antigen presenting cells (APC) like monocytes/macrophages and dendritic cells (DC). Polymorphnuclear cells develop in the bone marrow before migrating into the blood and contain neutrophils, basophils and eosinophils. Neutrophils are the most abundant type of leucocytes and normally the first to migrate from the bloodstream to the site of infection. Their main function is the elimination of the pathogen by phagocytosis, but next to the above mentioned antimicrobial peptides, they also produce reactive oxygen and nitrogen species with antimicrobial activity. Eosinophils and basophils both play a role in parasitic infections (e.g. in helminth infection) and allergies. They have limited phagocytic ability but act through the release of for example granule cationic proteins (eosinophils) or histamine (basophils). NK cells are lymphoid cells derived from bone marrow that share a common early progenitor with T cells and provide a fist line of defence against various viral infections and also play a role in the rejection of tumours. They can sense "altered self" or "missing self" and mediate target cell killing by apoptosis as their granules contain perforin and granzymes and they also bear FasL on their cell surface [reviewed in HAMERMAN JA ET AL. 2005]. Other cell types of the innate immune system sharing features with T cells are the NKT and γδT cells. NKT cells develop in the thymus, but their T cell receptor (TCR) is invariant and recognizes glycolipids presented by nonpolymorphic CD1d molecules. Due to the rearranging of their TCR genes and the potential to develop a memory phenotype, γδT cells bridge the innate with the adaptive immune system. Their recognition of common microbial antigens as well as the ability to bind to nonclassical self-MHC molecules and their rapid response suggest a role more akin to innate than adaptive immunity [reviewed in BORN WK ET AL. 2006]. Monocytes stem from haematopoietic stem cell precursors in the bone marrow and, once released into the blood stream, migrate rapidly to sites of infection and differentiate into macrophages and DC. Macrophages and DC phagocyte cellular debris or pathogens and present peptides from exogenous antigens on MHC class II molecules. In

the case of engulfed pathogens, meaning in the presence of a danger signal, they are activated and can, through the expression of costimulatory signals, prime and activate T cells.

In general cells of the innate immune system are the sentinels of the immune system to detect damage and danger. To do that cells of the innate immune system are equipped with pattern recognition receptors (PRR) that mostly detect foreign molecules except for some endogenous cellular products, like heat shock proteins or ATP, that signal tissue damage. PRR generally detect pathogen-associated molecular patterns (PAMP) or danger-associated molecular patterns (DAMP). They are either located on the cell surface (TLR, NLR), in the cytosol (NLR, RNA-helicase family) or within endosomes (TLR). Toll-like receptors (TLR) are known for about two decades and relatively well characterised. On the contrary, little is known about Nod-like receptors (NLR). Both types of PRR synergise to interpret danger and initiate appropriate actions. One medium which collects information from both groups of receptors and integrates them is a molecular scaffold complex called inflammasome. The most prominent output is the differential activation of precursor forms of IL-1b and IL-18. Whether IL-33 is also cleaved by the inflammasome is still unclear [SCHMITZ J ET AL. 2005]. Mutations or dysregulation of the PRR is known to the involved in the development of many pathologic conditions including autoimmunity and hypersusceptibility to infection [reviewed in CREAGH EM ET AL. 2006, FUKATA M ET AL. 2009].

To date, there are 10 (TLR1-10) known TLR in humans and 12 in mice (TLR1-9, TLR11-13). They are type I transmembrane glycoproteins. Their ligand binding domain consists of leucine-rich repeats (LRR) and are variable, whereas their signalling tail contains a highly conserved domain, the Toll-interleukin-1 receptor (TIR) domain. The TIR domain contains the binding motif for downstream elements. Signal transduction is mediated via two main pathways: (a) The MyD88-dependent pathway results in NFκ-B and AP-1 activation, (b) the TRIF-dependent pathway mainly activates expression of type I interferons (IFN). TLR detect the full spectrum of possible pathogen associated products, like microbial structures from bacteria, mycobacteria, RNA and DNA viruses, fungi or protozoans.

Plenty of NLR have been discovered, but only some are characterised. NALP1, NALP3 and IFAF mark the three different types of inflammasomes described in humans. The only member described in detail is the class II transactivator (CIITA) which is responsible for MHC II expression on APC [reviewed in MACH B ET AL. 1996]. APC are the cellular interface of the innate to the adaptive immune system. The innate immune system is in close contact with the adaptive one, instructing it on how to react to the danger present but also receiving directives itself.

Despite this intensive cross-talk between the two levels of immunity there is hardly any evidence for a genetic association with autoimmune disease [HUR JW ET AL. 2005, SHEEDY FJ ET AL. 2008]. This implies that genetic aberration of TLR and NLR does not seem to directly

cause any of the major autoimmune diseases. However, epigenetic involvement has been described. Most important for this study is the influence of certain TLR signalling on T cell lineage differentiation in the adaptive moiety of the immune system [FITZGERALD DC ET AL. 2007, LAMPROPOULOU V ET AL. 2008, FUKATA M ET AL. 2008].

Adaptive mechanisms

The adaptive arm of the immune system implements the power of somatic rearrangement to generate unlimited antigen recognition through a cellular system that grants the organism long lasting protection (immunological memory). Lymphocytes, which are divided into B and T cells, are its cellular players. B cells are of hematopoietic origin and are borne in the bone marrow where they differentiate into mature resting B cells. T cell precursors also originate in the bone marrow but migrate into the thymus to differentiate into T cells under the guidance of the local stroma. Each clone of B and T cells carries a unique receptor which enables it to detect foreign antigen. The structure of the B cell receptor (BCR) and the TCR is different to some extent as much as their competences and the modes of action of the two cell types.

B cells and T cells were discovered around the same time following a hypothesised cellular division of those that produce antibodies and originate from the *bursa fabricii* in birds (place of B cell haematopoiesis in birds; origin of the term B cell) and those responsible for a delayed hypersensitivity (DTH) response and are thymus derived [COOPER MD ET AL. 1965 & 1966].

B cells: Providing blood borne immunity

B cells provide the body's humoral immunity through the production of antigen specific antibodies. Antibodies are soluble BCR that B cells secrete in response to immunogenic stimulation. Antibodies/BCR consist of a highly diverse part which is randomly assembled by an error-prone genetic rearrangement of the V(D)J loci and a constant region (Fc) which defines the isotype of the antibody. In mice and humans five classes of antibodies exist: IgM, IgD, IgG, IgA and IgE. The isotype defines the function and localisation of the antibody. The induction of a B cell response culminates in a germinal centre reaction in secondary lymphatic tissue (SLT) leading to the immediate generation of antibody producing short lived plasma blasts and in the long run to affinity matured (product of somatic hypermutation), class switched, long-lived plasma cells (memory B cells) [reviewed in LEBIEN TW ET AL. 2008]. Secreted antibodies have several roles in immunity: (a) Neutralisation of antigen structures on the surface of pathogens. (b) Opsonisation of pathogens, which directs killing of the pathogen via phagocytosis or the complement system. (c) Marking pathogens for subsequent Fc-receptor mediated effector functions of other leukocytes [reviewed in NIMMERJAHN F ET AL. 2006]. For B cells to provide the full

range of humoral immunity T cell help in the germinal centres is required [reviewed in LINTERMAN MA ET AL. 2010]. There is a small fraction of epitopes that correspond to B cell function without T cell derived costimulation [reviewed in CALAME KL ET AL. 2003]. A great quantity of B cells generated is potentially autoreactive. Autoreactivity is purged by negative selection where autoreactive cells are deleted or by receptor editing where autoreactive cells change their BCR creating recessive tolerance [reviewed in PELANDA R ET AL. 2006, VON BOEHMER H ET AL. 2010]. B cells can be divided in at least three distinct lineages: B1a, B1b and B2. B1a are cells with little variability in their BCR. They are generated in the fetal liver but persist as minor population throughout life mostly producing IgM. B1b and B2 cells develop in the bone marrow and express a variable BCR. B2 cells are the "conventional" B cells (in this study called "B cells") as they are the majority and have the strongest impact on immunity [HARDY RR 2006].

T cells: Killers and Conductors

T cell development occurs in the thymus. Undifferentiated T cell precursors, thymocytes, mature by arranging and testing their T cell receptor (TCR). Generation of the TCR subunits involves steps homologous to those occurring during the generation of the BCR RAG-dependent genomic rearrangement of V(D)J elements. Variability is increased by a random insertion of nucleotides at most joining sites (N-regions). The TCR is a heterodimer consisting of two transmembrane proteins, either an alpha and beta- or a gamma and delta chain. Either expression determines the lineage fate [reviewed in VON BOEHMER H ET AL. 2010]: Successful expression of the pre-TCR (upon *tcrb* rearrangement), which signals ligand-independently in a cell-autonomous fashion, together with Notch signalling commits thymocytes to the TCRαβ lineage [WASHBURN T ET AL. 1997, YAMASAKI S ET AL. 2007]. Rearrangement of the *tcrg* and *tcrd* loci commits them to the TCRγδ lineage which only happens to a minor fraction of the precursor cells. TCR specificity also directs the thymocytes towards the $CD4^+$, $CD8^+$ or natural regulatory T cell (nT_{reg}) lineage. The TCR specificity is unpredictable and has potential binding capabilities towards any possible antigen including self antigens. To diminish the great potential for auto-aggressive T cells, nascent T cells have to reveal their binding potential to MHC:self-peptide complexes (spMHC) to antigen-presenting cells. The thymic positive and negative selection processes eliminate those T cell clones from the T cell repertoire that do not (MHC restriction) and those that bind strongly to spMHC (recessive/central tolerance) [reviewed in VON BOEHMER H ET AL. 2008 AND 2010]. Most of the selection takes place in the medullary region of the thymus where peripheral self-antigens are presented on MHC by immigrating DC [BONASIO R ET AL. 2006] and/or medullary epithelial cells by AIRE-dependent ectopic gene expression [ANDERSON MS ET AL. 2002]. There is a lifelong need to establish tolerance to self as lymphocytes are continuously generated.

The weak interaction between the TCR and spMHC that is necessary to pass positive selection is usually not sufficient to elicit an autoimmune response but acts as a survival

signal that is crucial in T cell homeostasis. As naive T cells have to compete throughout their life for spMHC contact this favours a most diverse repertoire of TCR. Besides the weak TCR signalling common γ chain (γc) cytokines (IL-2, IL-7 and IL-15) and their receptors on the T cells regulate T cell survival and expansion [reviewed in SURH CD ET AL. 2008].

$CD8^+$ T cells are MHC I restricted cytotoxic lymphocytes (CTL). MHC I is expressed by almost all nucleated cells in the body. At certain immuno-privileged sites MHC I expression can be restrained (e.g. testis, eye, CNS) [reviewed in NIEDERKORN JY 2006]. MHC I presents cell endogenous peptides thereby depicting the full profile of the cell's biosynthesis. Many intracellular pathogens (e.g. viruses) are dependent on using the biosynthesis machinery of the host cell to produce pathogen associated factors. Since every cell of the body has to account for the proteins they produce, MHC:foreign-peptide complexes (pMHC) would be presented on the surface of an infected cell. Primed CTL that home into peripheral tissue are activated by those pMHC on the surface of any cell and in turn start their cytotoxic response which destroys the target cell and contributes to shaping the cytokine milieu at the site of infection. The former is performed by soluble cytotoxins (perforin and granzymes) and membrane-bound pro-apoptotic factors (e.g. FasL), the latter by secretion of proinflammatory cytokines (e.g. IFNγ, TNFα, IL-17A) [WALTER U ET AL. 2005].

Due to their destructive capacity their priming has to be tightly controlled. A CTL can only become active when a $CD4^+$ T cell counterpart of the CTL is activated and subsequently licenses a DC that cross-presents their cognate antigen on MHC I. Only then the DC can give sufficient costimulatory signal to prime the CTL. T_H cell-derived IL-2 then becomes crucial for clonal expansion of the CTL [CHO JH ET AL. 2010]. Cross-presentation is the unconventional processing of exogenous antigen via the MHC I pathway normally restricted to endogenous antigens [HEATH WR ET AL. 2001]. As well as providing a mechanism for generating immunity to intracellular infections, cross-presentation can also induce tolerance of CTL to self-antigens when they are cross-presented (cross-tolerance) in the thymus or the periphery [VON BOEHMER H ET AL. 1986].

$CD4^+$ T cells that leave the thymus can be divided into distinct lineages: T helper cells (T_H cells), natural regulatory cells and natural killer T cells (NKT cells). T_H cells are the central cell type in adaptive immunity. They contain the ability to: (a) help B cells to produce antibodies, (b) regulate CTL activation, (c) recruit granulocytes to the site of inflammation, (d) stimulate anti-microbial activity by macrophages and (e) orchestrate the overall cellular immune response by the secretion of various cytokines. All their functions are induced in a MHC II restricted manner. They mainly exert their functions through secreting cytokines that direct inflammation, leukocyte migration and the direction of the overall immune-response. Loss, misguidance or uncontrolled autoreactivity of T_H cell can have a detrimental effect on the organism leading to immunoinsufficiency, allergy or autoimmunity respectively. They are the focus of interest in this study and will be further specified in the following paragraphs.

T cell help in different flavours: T_H cell effector types

Upon recognition of cognate antigen presented by an activated professional APC T cells become polarised towards an effector type depending on the quality of the priming immune synapse and the cytokines present. The classic paradigm proposed by MOSMANN and COFFMAN in 1986, stems from the finding that long-term T_H effector cell lines can be clearly divided into two subtypes base of the expression of distinct sets of cytokines. T_H1 cells were defined by their primary production of IFNγ and IL‑2 while T_H2 cells were defined by IL‑4 and IL‑5 secretion [MOSMANN TR ET AL. 1986].

Since then considerable effort has been made to proof or disproof the two effector types of T_H cells to be discrete lineages and to understand their distinct function in an immune response. By now the T_H effector types have been confirmed in mice and in humans [DEL PRETE GF ET AL. 1991] and their imprint on the immune response has been clarified. T_H1 cells mostly direct cell-mediated immunity and are the prime initiators of a delayed hypersensitivity response. T_H2 cells mostly regulate B cell activity and humoral immunity. IgE responses and eosinophil activity are greatly controlled by T_H2 cells which makes them the driving force in allergic reactions. Concerning the expression of cell surface markers there is a strong trend to T_H1 cell expressing IL-12Rβ2 chain, IL-18 receptor, P-selectin glycoprotein ligand-1, Chandra, and the CXCR3 and CXCR5 chemokine receptors, whereas T_H2 typically express the chemokine receptors CCR3, CCR5, and CCR8, and inducible costimulatory molecule (ICOS) [reviewed in HO IC ET AL. 2002].

The priming of naive T cells by an APC can be divided into three types of signals that are integrated by the T cell to define the effector response that it will pursue. The first signal is the TCR-MHC interaction; it is mandatory to allow any kind of T cell activation. The signal strength that the T cell receives depends on the avidity of the interaction. The avidity is a function of the affinity of each single molecular interaction and the number of interacting partners, which depends on the number of this specific MHC:peptide complexes presented by the APC. Furthermore, a directed plasma membrane organisation can influence the avidity of molecular interactions. Floating topological entities on the surface of cells (lipid rafts) concentrate proteins on the cell surface. This can be essential for a protein's function due to an increase in interaction avidity. TCR signalling is to some extent dependent on the formation of a functional unity in lipid rafts [XAVLER R ET AL. 1998]. Whether lipid rafts play a role in MHC II presentation on APC is a matter of debate. However, it could increase local MHC:peptide concentration on the cell surface which could be a mechanism to allow efficient T cell activation with infrequent MHC:peptide complexes. The degree of avidity influences the behaviour and development of the activated T cell [BOYTON RJ ET AL. 2002].

The second signal allows the T cell to know whether it faces a foreign- or a self-peptide presented by the APC. This is achieved by a set of membrane bound costimulatory

molecules of which expression is only induced on an APC, when it has acquired an antigen in the context of "danger". The sentinels of "danger" are mostly innate immune receptors expressed on the surface of peripheral APC. Hence the notion that APC translate between innate and adaptive immunity [STEINMAN RM ET AL. 2006]. The lack of costimulation during priming directs the T cell to become anergic or possibly die by apoptosis. After successful initial activation the T cell response is regulated by coinhibitory signals. Consequently, T cell differentiation and expansion depend on the balance between costimulatory and coinhibitory signals. The most prominent costimulatory interaction takes place between two members of the B7-family, B7-1 (CD80) and B7-2 (CD86) expressed on professional APC and CD28 expressed on the T cell. For coinhibitory signalling these B7 members interact with CTLA-4, which can be upregulated on T cells. In the last ten years many more B7 members on the APC side and counterparts on the T cell side have been described [DRIESSENS G ET AL. 2009]. T cells also provide stimulatory signals for APC through expression of CD40L (CD154) which interacts with its receptor CD40 on APC. CD40 signalling leads to cell activation, survival and upregulation of costimulatory molecules [QUEZADA SA ET AL. 2004].

The "third signal" summarises the contribution of soluble mediators the T cell priming and polarisation. The cytokine milieu at the time of T cell-APC cross-talk is probably mostly shaped by the APC that activate the T cells. Nonetheless, also surrounding cells like activated stroma participate. Cytokines are a powerful tool to shape a developing immune response. The first description of *in vitro* differentiation of T_H cell was reported in 1990. Stimulation of naive T_H cells with IL-4 turned them into IL-4 producing effector cells [LE GROS G ET AL. 1990, SWAIN SL ET AL. 1990]. In 1993 the first T_H1 cells were generated by stimulation with IL-12 and IFNγ [HSIEH CS ET AL. 1993]. Subsequent *in vivo* investigations confirmed these cytokine to be essential for T_H polarisation. It was shown that T_H effector cell lineage commitment employs autocrine loops for 'self-propagation' and reciprocal inhibition via their signature cytokines as driving forces of polarisation. The T_H1 and T_H2 differentiation processes seem to work with a similar logic: Both contain a T cell endogenous factor, INFγ in the case of T_H1 and IL-4 in the case of T_H2, which amplifies polarisation via a positive feedback loop. Anti-IFNγ and anti-IL-4 treatment is sufficient to skew differentiation accordingly into the opposite direction. A difference in the global scheme of polarisation exists between the two lineages, in the way that there is an exogenous factor in the T_H1 differentiation, namely IL-12, which is mostly produced by APC at the priming event. It is important in initiating IFNγ secretion by nascent T_H1 effector cells. To date no analogue to IL-12 has been discovered in T_H2 differentiation. An analogous set of effector subsets appears to exist also in the $CD8^+$ T cell lineage but is not as well understood as their T_H cell counterparts [YEN HR ET AL. 2009].

A major premise for the classification into cell lineages is the existence of transcription factors (TF) that drive a singular cell fate, which upon activation leads to a stable lineage commitment. TF function in general is dependent on its concentration, post-translational

modification and subcellular localisation. All three aspects can be independently controlled. Roughly ten years after MOSMANN proposed the dichotomy in the TH effector line the two groups of FLAVELL and RAY presented GATA-3 to be the one TF necessary and sufficient for a TH cell to develop TH2 characteristics [ZHENG W ET AL. 1997, ZHANG DH ET AL. 1997]. Three years later in 2000 GLIMCHER and SZABO succeeded in determining the master TF of the TH1 lineage, the "T-box expressed in T cells" TF (T-bet, encoded by *tbx21* in the mouse) [SZABO SJ ET AL. 2000].

Upon TCR stimulation naive TH cells turn into an activated state driven by TFs like nuclear factor of activated T cells (NFAT). Costimulation via CD28 induces the Fos-jun heterodimer (AP-1). The NFAT-AP-1 complex triggers IL-2 and IL-2R expression. By contrast NFAT activity without AP-1 induces anergy respective to spMHC engagement without costimmunlation [WU Y ET AL. 2006, RUDENSKY AY 2006]. IL-2 signalling upregulates expression of the signal transducer and activator of transcription (STAT) 5 in activated TH cell and initiates the cell entering the cell cycle. Interestingly STAT5 can promote *ifng* and *il4* expression [TAYLOR DK ET AL. 2006]. At that point the cytokine milieu becomes most influential on the cell fate decision. In the case of TH1 polarisation T-bet is induced mainly by STAT1 and STAT4. STAT1 is upregulated upon type I and type II IFN receptor engagement and via IL-27 signalling. STAT4 in the TH1 context is driven by IL-12 signalling [THIEU VT ET AL. 2008]. T-bet, H2.0-like homeobox (HLX), runt-related TF (RUNX) 3 and STAT4 bind to and activate *ifng*, which forms a positive feedback loop by the autocrine induction of STAT1. Reciprocal inhibition is facilitated through binding and repression of *il4* by RUNX3 and T-bet [reviewed in AMSEN D ET AL. 2009A]. GATA3 in TH2 differentiation is induced by STAT6 which is dependent on IL-4 signalling. Although still a matter of investigation initial IL-4 could be contributed by activated memory $CD4^+$ T cells or basophils [TANAKA S ET AL. 2006, SOKOL CL ET AL. 2008]. GATA3, STAT6 and Gfi-1 induce TH2 signature cytokine transcription. Primarily GATA3 stabilises TH2 differentiation its autoactivation and by activation of STAT6 and IL-4. Reciprocal inhibition of the TH1 lineage is performed by STAT6- and GATA3-dependent antagonisation of IFNγ expression [OUYANG W ET AL. 2000]. Inhibition of TH17 and T_{reg} differentiation is performed by Gfi-1 [ZHU J ET AL. 2009]. All components of cellular signal transduction are woven into a multilevel network of conditional connectivity which serves differential functions dependent on the context. Accordingly, neither T-bet nor GATA3 only control TH effector cell differentiation but serve alternative purposes in other developmental contexts [reviewed in GLIMCHER L 2007 and HO IC ET AL 2009].

More recently an independent signalling pathway has become appreciated that can most likely promote TH1 as well as TH2 polarisation: The Notch signalling in activated T cells [AMSEN D ET AL. 2004, reviewed in AMSEN D 2009A]. Even though IL-12 and IL-4 are crucial driving forces of TH1 and TH2 differentiation respectively, there is clear evidence that they are not completely essential but mainly amplifying forces [AMSEN D ET AL. 2009B]. TH1-like

responses to all kinds of antigens (e.g. against viruses, bacteria, in diabetes etc.) have been observed irrespective of the absence of IL-12/12R signalling components. Similarly TH2 responses can be active *in vivo* while lacking IL-4/4R signalling. Notably, in particular helminth, which are amongst the strongest inducers of IL-4 production seem to mount a similar TH2 response in the absence of IL-4 as compared to in the wild type situation [KING SB ET AL. 2008]. The notion of either IL-12 or IL-4 being essential factors for lineage commitment is mostly fuelled by *in vitro* findings but is in all not evidentiary [KAPLAN MH ET AL. 1998, JANKOVIC D ET AL. 2000]. Notch signalling in TH1 polarisation is still a matter of debate since besides a lot of descriptive, correlative data suggesting a connection the results from genetic studies were not completely cogent. Several gain-of-function studies demonstrated that expression of members of the Notch ligand family of DLL proteins on APCs and Notch on T cells induces TH1 lineage commitment [MAEKAWA Y ET AL 2003, SUN J ET AL. 2008]. Loss-of-function studies paint a more complex picture on Notch signalling within the T cell as the lack of Notch signalling components does not in all cases lead to a defect in TH1 polarisation. Altogether it seems that the influence of Notch signalling on TH1 is greater in IL-12 independent TH1 responses [SKOKOS D ET AL. 2007]. Whether the overall effect is due to a directly TH1 promoting effect or due to an inhibition of IL-4 dependent GATA3 regulation is unclear. A significantly stronger and more probative body of evidence exists that implicates Notch signalling in TH2 differentiation. Whereas expression of DLL ligands is associated with TH1 differentiation, expression on APC of Jagged ligands is associated with TH2 responses, and ectopic activity of Jagged1 can promote TH2 cell differentiation [AMSEN D ET AL. 2004, ELYAMAN W ET AL. 2007]. Basically all gain-of-function and loss-of-function studies performed on the subject point towards a critical impact of Notch. How exactly Notch acts however is controversial [reviewed in RADTKE F ET AL. 2010]. *In silico* analysis, chromatin immunoprecipitation studies as well as *in vivo* studies have confirmed direct binding of Notch signalling components to promoter elements of *il4* and *gata3* [AMSEN D ET AL. 2004, TANIGAKI K ET AL. 2004, FANG TC ET AL. 2007]. It is possible that IL-4 expression is actually downstream of Notch which would complement well with the fact that high amounts of exogenous IL-4 are able to override the requirement of Notch in TH2 differentiation. Possibly Notch and GATA3 synergise in the promotion of TH2 effector lineage commitment [FANG TC ET AL. 2007]. How Notch decides whether to drive TH1 or TH2 in not understood. To complicate matters even more, Notch has also been implicated in the induction of tolerance by promoting T_{reg} differentiation in overexpression models [KARED H ET AL. 2006, SAMON JB ET AL. 2008].

Apart from gene regulation via TFs which shapes the expression profile of a cell lineage or transitional state there are at least three other major mechanism that regulate gene expression: (a) The intrinsic stability of transcribed mRNAs encoded in their 3'-untranslate regions (UTR). It is often employed to regulate the extend and the timing of a response [HAO S ET AL. 2009]. (b) The 3'-UTR can also be target of an RNA-induced silencing complex lead by microRNA. The knowledge on microRNA stems from pioneering studies in plants and

nematodes [reviewed in SINGH SK ET AL. 2008]. Although it is still a very young field of research it has become apparent that silencing microRNA is crucially involved in cellular fate decisions in the hematopoietic system [reviewed in BALTIMORE D ET AL. 2008]. (c) Epigenetic changes affect the context in which TF function. The changes are on the level of DNA, histones and chromatin structure and constitute a form of inheritable developmental "memory" that consolidates a lineage decision in the long term. This aspect will be further elaborated at a later point in this chapter (cf. "Introduction: Lineage plasticity").

Regulatory T_H cells: Immunity *versus* tolerance

Proposed already in the 80s by SAKAGUCHI [SAKAGUCHI S ET AL. 1985 & 1995] regulatory T cells (T_{reg}) officially joined in the ranks of the T_H effector types in 2003 when the "master regulator" of T_{reg}, the forkhead box P3 (FoxP3) TF, was discovered by three independent groups [HORI S ET AL. 2003, KHATTRI R ET AL. 2003, FONTENOT JD ET AL. 2003]. In mice a point mutation *in foxp3* results in a lethal $CD4^+$ T cell–mediated lymphoproliferative disease characterised by cachexia and multiorgan lymphocytic infiltrates (*scurfy* strain) [BRUNKOW ME ET AL. 2001]. The mutation in the human homologue causes global immune dysregulation with autoimmune endocrinopathy, early-onset type 1 diabetes and thyroiditis and in some cases manifestations of severe atopy including eczema, food allergy and eosinophilic inflammation (IPEX; also called X-linked autoimmunity-allergic dysregulation syndrome, XLAAD) [CHATILA TA ET AL. 2000]. T_{reg} protect the organism from self destruction. Therefore, T_{reg}-enforced tolerance counteracts the development of autoimmunity as well as counter-regulates protective immune responses. The lineage of T_{reg} can be subdivided into natural T_{reg} (nT_{reg}) which are fully differentiated in the thymus and induced T_{reg} (iT_{reg}) which develop in the periphery. Both finally end up in the periphery where they make up 5-10% of the $CD4^+$ T cell pool in mice and humans. T_{reg} in the periphery are $CD25^+$ (IL-2Rα, high affinity receptor subunit for IL-2) $CD127^-$ (IL-7α receptor subunit) $FoxP3^+$. This reflects their dependence on IL-2 and not on IL-7 signalling for survival [reviewed in OHKURA N ET AL. 2010]. Activated human T cells also express CD25 and transiently FoxP3 but to a lesser extent. Instructed in the thymus alongside all other developing T cells, they are designed to keep autoreactive T cells in check that constantly pass thymic selection [reviewed in FEHÉRVARI Z ET AL. 2004]. Hence, CD25-depleted mature thymocytes or splenocytes adoptively transferred into syngeneic T cell-deficient hosts produce severe autoimmunity [ITOH M ET AL. 1999]. Taken together, peripheral APC-T cell interaction can either prime T_H cells to become T_H1, T_H2, T_H17 and/or iT_{reg} effector cells and/or activate nT_{reg}. The generation and survival of T_{reg} is TGFβ dependent [CHEN W ET AL. 2003, MARIE JC ET AL. 2005, LI MO ET AL. 2006]. In naive T_H cells Foxp3 can be induced by TGFβ and IL-2, as well as retinoic acid (RA) [ELIAS KM ET AL. 2008, TANG Q ET AL. 2008]. In terms of lineage induction in the periphery T_{reg} and T_H17 are somewhat linked by their common dependence on TGFβ signalling (cf. "Introduction: T_H17 cells"). In the periphery

nT_{reg} and iT_{reg} are the primary source of TGFβ. Therefore the status of T_{reg} might be critical in the differentiation of both iT_{reg} and T_H17 cells. Whether iT_{reg} and nT_{reg} contribute differently to peripheral tolerance is not clear [CHEN W ET AL. 2003]. T_H1 and T_H2 are subjects of $FoxP3^+$ T_{reg} suppression [KIM JM ET AL. 2007]. Recent data suggest a similar effect on T_H17 cells [CHAUDHRY A ET AL. 2009]. Besides T cells also DC, macrophages, NK cells, NKT cells and B cells are being regulated by T_{reg} [reviewed in SAKAGUCHI S ET AL. 2008]. Similarly as for the respective TF in the T_H1 and T_H2 lineages the signature TF of T_{reg} FoxP3, stabilises T_{reg} commitment by increasing its own expression [GAVIN MA ET AL. 2007]. Nevertheless, current data does not suggest a critical role for FoxP3 in the initial T_{reg} development in the thymus; FoxP3-deficiency allows the generation of T_{reg}-like cells but rather prevents T_{reg} lineage establishment and function [LIN W ET AL. 2007]. It still has to be established which signalling code induces FoxP3 expression. The TCR signalling is certainly a crucial part of it. It has been shown in TCR transgenic models that thymocytes have to be allowed to express endogenous TCRα-chains to develop into T_{reg} [ITOH M ET AL. 1999]. Accordingly, RAG^{null} mutant TCR transgenic mice (e.g. OT II) lack $FoxP3^+$ T_{reg}. There is a host of data pointing out different aspects of FoxP3 induction, which as a whole are not yet fully conclusive. What is, however, collectively suggested is that the TCR specificity has an instructive influence in the induction of T_{reg} fate in thymocytes [reviewed in WING K ET AL. 2010]. FoxP3 is first detected in the late double positive state ($CD4^+$ $CD8^+$, DP). Once T_{reg} are fully mature they can migrate to the sites of inflammation after antigen-specific stimulation by an APC. Gene transcription analysis reveals that the expression profile of T_{reg} in different tissues or inflammatory context differs from each other. This might reflect the differential activity of a wide range of regulatory effector responses. There are many known modes of action that can be induced; some are cell-contact mediated (e.g. functional modulation by means of CD39, CD73 and LAG-3, or killing of APCs or responder T cells by means of granzyme and perforin), others involve secretion of soluble factors (secretion of immunosuppressive cytokines such as IL-10, transforming growth factor β (TGFβ), IL-35 and galectin-1, or deprivation of cytokines (e.g. IL-2) necessary for the expansion and/or survival of responder T cells) [WING K ET AL. 2010].

<u>T_H17 cells</u>

The notion that there is an effector T_H cell population that is found in similar context but different in its effector potential to T_H1 cells originates from two early papers in 1999 and 2000. In 1999 AARVAK ET AL. analysed T_H cell effector lines from the synovial tissue/fluid from rheumatoid arthritis patients and found that those lines which were categorised as T_H1 or T_H0 according to their cytokine profile could be split in those that do and those that do not co-produce IL-17A [AARVAK ET AL. 1999]. In 2000 INFANTE-DUARTE ET AL. published an *in vitro* differentiation study on primary mouse and human T_H cells that suggested that IL-17A producing T_H cells are distinctly different to T_H1 and T_H2. The newly supposed subset

was characterised by IL-17A, TNFα and GM-CSF expression. IL-6 was found to promote IL-17A expression in this system [INFANTE-DUARTE ET AL. 2000]. Five years had to pass until the impact of these findings started to unfold. In 2005 the groups of WEAVER and DONG both described the *in vitro* generation of IL-17A producing TH cells and their lineage characteristics, as they develop independently of the TH1 and TH2 transcription factors and are inhibited in their differentiation by TH1 and TH2 signature cytokines [reviewed in STUMHOFER JS ET AL. 2005]. The new TH effector lineage was termed TH17. Both papers as well as others implicated a significant role of TH17 cells in autoimmune pathogenesis and a clear dependence on IL-23 for their differentiation *in vivo* [AGGARWAL S ET AL. 2003, LANGRISH CL ET AL. 2005]. In 2006 VELDHOEN ET AL. pinpointed TGFβ and IL-6 as the necessary and sufficient initiators of generation of TH17 cells in *in vitro* polarisation assays. It was a surprising finding that IL-23 was not necessary for *de novo* genertion [VELDHOEN M ET AL. 2006]. In the same year an exclusive TF that controlled all known features of TH17 cells was found, the retinoid-related orphan receptor isoform γt (RORγt, one gene product of the *rorc* gene locus) [IVANOV II ET AL. 2006]. RORγt expression is sufficient to induce IL-17A expression in the absence of cytokines. Chromatin immunoprecipitation assays (ChIP) have suggested that *il17a* is a direct target [ZHANG F ET AL. 2008]. Mice deficient in RORγt are largely deficient in TH17 cells, yet a small fraction of those cells does develop. The suggested redundancy in the system seems to be due to another member of the same receptor family, RORα, which has been identified to partner with RORγt in TH17 development. Although RORα contribution appears to be dispensable for TH17 differentiation, its enforced expression in Jurkat T cells is sufficient to drive IL-17A and IL-22 expression [YANG XO ET AL. 2008, DU J ET AL. 2008]. Double deficiency of RORα and γ abolishes IL-17A and -17F expression by activated TH cells completely [YANG XO ET AL. 2008]. The ROR family of nuclear receptors consists of three members and constitutes diverse functions in transcriptional regulation. RORα is expressed in a variety of different cell types governing many aspects of development and metabolism in muscle, bone and neuronal tissue. RORβ is mostly found in the CNS - often connected with tissue controlling the circadian rhythm [JETTEN AM 2009]. Besides its essential function in TH17 differentiation RORγ plays a crucial role in organogenesis of secondary lymphatic tissue (SLT) and thymopoiesis [SUN Z ET AL. 2000, KUREBAYASHI S ET AL. 2000, EBERL G ET AL. 2004].

The discovery of a discrete transcriptional pattern and regulation established TH17 cells as a lineage. The last five years have seen an incredible amount of data being produced on the subject which forms a rich picture of the new TH effector type. Nevertheless it still falls short in being fully conclusive on many major aspects of their differentiation, regulation and function. TH17 cells are characterised by the expression of IL-17A, IL-17F, IL-22, IL-21, TNFα and GM-CSF [AGGARWAL S ET AL. 2003, reviewed in WEAVER CT ET AL. 2007]. However, the expression of the cytokines is not necessarily simultaneous, but their individual expression depends on factors that are still a matter of investigation. In humans another factor has been recently added as a signature cytokine, IL-26, which does not have a known orthologue in

mice [WILSON NJ ET AL. 2007]. Regarding surface receptors, mature TH17 cells typically express IL-23R, IL-1R1 and IL-18Rα. Unfortunately, TGFβR expression is not sufficiently analysed on TH17 cells. On human TH17 cells CCR6 and 4 are additional lineage markers. They enable human TH17 to migrate to the site of inflammation [ACOSTA-RODRIGUEZ EV ET AL. 2007]. Interestingly, in the skin and the mucosal tissue the CCR6 agonist, CCL20, is constitutively expressed. Cytokine secretion of TH17 cell in the inflamed mucosa leads to the induction of more CCR6 agonists (e.g. β-defensins) [KAO CY ET AL. 2004 & 2005].

The overall picture suggests that TH17 cells play a vital role in the immune response against extracellular bacteria and fungi, where granulocyte recruitment is highly protective [reviewed in SALLUSTO F ET AL. 2009, PECK A ET AL. 2010]. KOLLS and colleagues were the first to show their role in infection in a model of *Klebsiella pneumoniae* infection by demonstrating the importance of the IL-23/IL-17A-axis for immunity against the pathogen [HAPPEL KI ET AL. 2005]. By now many bacteria, mycobacteria and fungi turned out to be susceptible targets of a TH17 dominated immune response. Although the great majority, not all of the bacteria that TH17 cells are effective against are extracellular which results in an overlap of TH1 and TH17 function in infection [reviewed in CURTIS MM ET AL. 2009]. Furthermore, the protective effect was consistently found to be more modest for intracellular pathogens [SCHULZ SM ET AL. 2008]. Nevertheless, it is compelling that TH17 cells have filled a niche in the immunity to infection that was not fully covered before by TH1 and TH2 effector cells. Most of the anti-microbial effect of TH17 cells is mediated through the known TH17 effector cytokines.

T_H17 and T_{reg} cell fates compete in the presence of TGFβ

While IL-23 *in vivo* and *in vitro* has IL-17A, IL-17F and IL-22 promoting effects, the lineage commitment of naïve T cells appears to be IL-23 independent *in vitro*. Several groups addressed the question which factors trigger the generation of TH17 cells from naïve precursors. IL-6 and TGFβ, two cytokines with in many ways opposing effects, were found to direct the polarisation of TH17 cells *in vitro* [MANGAN PR ET AL. 2006, BETTELLI E ET AL. 2006, VELDHOEN M ET AL. 2006]. TGFβ signalling is involved in a broad range of processes, including embryogenesis, cell proliferation, apoptosis and the immune response. There are three homologous TGFβ isoforms in mammals, TGFβ1, TGFβ2 and TGFβ3, encoded by different genes. TGFβ1 (in this study called TGFβ) is the predominant isoform expressed in the immune system, but all three isoforms have similar properties *in vitro* [GOVINDEN R ET AL. 2003]. TGFβ is produced in its latent form and needs to be activated. This is achieved either by proteolytic degradation or by conformational changes of latency-associated protein (LAP), which is associated with TGFβ in a heterotetrameric form and constrains the binding of the TGFβ homodimer to its receptor. TGFβ signalling promotes $CD8^+$ T cell and NKT cell development and inhibits the proliferation of thymic regulatory T cells. In peripheral tissues, TGFβ inhibits T cell proliferation, activation and effector T cell differentiation and on the other hand maintains T_{reg} cells [LI MO ET AL. 2006; MARIE JC ET AL. 2006]. TGFβ in the control

of immune homeostasis is not fully understood because of its pleiotropic action and its severe phenotypes when blocked of overexpressed *in vivo*. Mice without TGFβ develop early onset autoimmune disease with a characteristic severe multiorgan pathology which leads to their death at a few weeks of age [KULKARNI AB ET AL. 1993, SHULL MM ET AL. 1992]. All data generated with conditional deletion of either TGFβ itself or its receptor as well as the introduction of a transgenic dominant negative form of truncated TGFβ point towards a clear role of TGFβ in TH17 differentiation also *in vivo*; however, whether the deficit in the TH17 lineage in those mice is due to a direct effect on the developing T cells or an indirect phenomenon cannot be easily resolved with the genetic tools published so far. Converse to the null mutant mice, TGFβ conditional overexpression models show an increase in TH17 cells which marks TGFβ as a beneficial factor for TH17 differentiation [BETTELLI E ET AL. 2006]. Our laboratory is currently working with a tamoxifen inducible conditional TGFβRII^{null} mutant restricted to the CD4 lineage. This strain did not show any sign of autoimmunity when treated for up to 150 days. Thus, this is a promising experimental system to investigate TGFβ signalling deficiency in an otherwise healthy organism [SLEDZINSKA A ET AL. (in preparation)].

The signalling pathways of TGFβ are not well understood. It is known that TGFβ promotes TH17 development on at least three different levels: (a) TH1/TH2 inhibition, (b) RORγt transcription, (c) SOCS3 inhibition, which will be discussed in the following paragraph.

The inhibition of TH1 and TH2 differentiation is facilitated by down-regulating T-bet and GATA3 respectively [GORELIK L ET AL. 2000 & 2002]. Conversely, TGFβ upregulates both FoxP3- and RORγt-expression establishing a competitive antagonism which resolves contingent upon proinflammatory co-signals. In human umbilical cord blood T cells it has also been suggested that TGFβ downregulates T-bet activity [SANTARLASCI V ET AL. 2009]. In the FoxP3/RORγt double positive stage RORγt expression is progressively extinguished directing cell fate towards the T_{reg} lineage [YANG XO ET AL. 2008,]. This is due to a dominant inhibition of RORγt transcriptional activity by FoxP3. The inhibition is at least in part facilitated by the physical interaction between RORγt, RORα and FoxP3 [ZHOU L ET AL. 2008, DU J ET AL. 2008, ICHIYAMA K ET AL. 2008]. However, the dominance of FoxP3 can be overturned by a strong pro-inflammatory signal that utilises STAT3 activation. Lack of FoxP3 at that point does not lead to T_{reg} differentiation in the absence of STAT3 signals [YANG XO ET AL. 2008]. IL-6, IL-1β, IL-21 and IL-23 all preferentially signal via Janus family kinases leading to STAT3 activation and are thus TH17 driving co-factors [O'SHEA JJ ET AL. 2008]. IL-6 is supposed to be the major factors involved in the differentiation of naive TH cells since the IL-23R and IL-1R are not yet expressed on these cells and IL-21 is mostly produced by differentiated TH17 cells. STAT3 is essential for TH17 cell development [YANG XO ET AL.2007, NISHIHARA M ET AL. 2007, HARRIS TJ ET AL. 2007, LIU X ET AL. 2008]. STAT3 like RORγt binds to the *il17a/f* promoter region as is shown by ChIP [CHEN Z ET AL. 2006]. Both TF might also synergistically bind to regulative *cis*-elements of the *il17a/f* loci [ZHOU L

ET AL. 2007]. Forced expression of RORγt partially recovers IL-17A expression suggesting that RORγt acts downstream of STAT3 [ZHOU L ET AL. 2009]. Further functions of STAT3 in the context of TH17 are the upregulation of IL-21 (direct binding to *il21* locus) via IL-6 signalling [WEI L ET AL. 2007] and also the subsequent upregulation of IL-23R upon IL-6 and IL-21 signalling seems to be STAT3-dependent [ZHOU L ET AL. 2007, NURIEVA R ET AL. 2007]. Embedded in the JAK-STAT3 pathway is a further level of control in the form of the co-activation of the suppressor of cytokine signalling 1 and 3 (SOCS), an immediate negative feedback loop of STAT1 and 3 activation [LANG R ET AL. 2003, CROKER BA ET AL. 2003]. SOCS1 inhibits IFNγ and IL-4 which indirectly promotes TH17 development [TANAKA K ET AL. 2008]. Deletion of SOCS1 in T cells primarily leads to an increase of TH1 cells and a defect in TH17 development. In contrast, SOCS3 deficiency in T cells results in an increase of TH17 cells. To a certain extend, TH17 development appears to depend on the inhibition of SOCS3. TGFβ signalling inhibits SOCS3 expression and thus allows optimal TH17 differentiation ensuring a context dependent response to the IL-6/IL-21/IL-23 signalling [QIN H ET AL. 2009]. Nevertheless, STAT3 is also driving the anti-inflammatory program of IL-10 signalling. The mechanism behind whether STAT3 acts pro- or anti-inflammatory has not been resolved to date. EL KASMI ET AL. showed that also IL-22 signalling, which is mediated via STAT3 but in most physiologic contexts results in a proinflammatory transcriptional program, can induce an IL-10-like anti-inflammatory program in macrophages. Even IL-6, which is a purely proinflammatory cytokine, can induce an STAT3 mediated anti-inflammatory program when the SOCS3 negative feedback is completely lifted from its STAT3 signalling [EL KASMI KC ET AL. 2006]. STAT3 is thus an ambiguous signalling pathway that can either drive a highly proinflammatory or an inflammatory program which could explain the ambiguity of IL-22 responses (cf. "Introduction: IL-23 induced effector cytokines").

Endogenous and exogenous modulatory factors in effector type decision

The choice whether the nascent TH cell develops a T_{reg} or a TH17 phenotype does not depend on FoxP3 and RORγt alone but on the complex signalling pattern that influences the activity of either TF. The following are mechanistic examples of how this modulation could be achieved.

TGFβ induces the phosphorylation of Smad2 and 3 which can bind to Smad4 forming a complex that translocates into the nucleus to regulate TGFβ induced gene transcription. Interestingly, while Smad3 is essential for T_{reg} and TH17 differentiation, Smad4 is only involved in T_{reg} differentiation [YANG XO ET AL. 2008]. More recently, Smad3 was found to directly inhibit RORγt transcriptional activity [MARTINEZ GJ ET AL. 2009] as one means to promote FoxP3 activity. This is one of the few evidences addressing the differential role of TGFβ signalling in cell lineage commitment. The proinflammatory signal, which is required as a co-factor for TH17 differentiation, supposedly reliefs the inhibitory dominance of FoxP3 on RORγt. This seems to be irrespective of any major changes in the transcription levels of

RORγt suggesting post-transcriptional mechanisms. RUNX1 is a transcriptional co-factor in T_H17 differentiation. It binds to the RORγt/RORα complex. It is speculated that it might be involved in the modification of the RORγt-FoxP3 interaction to prevent FoxP3-mediated inhibition [ZHANG F ET AL. 2008, REVIEWED IN ZHOU LET AL. 2009]. RUNX1, however, appears to be also involved in T_{reg} differentiation [ONO M ET AL. 2007]. The control over proinflammatory signals is not only by the sender but also on the side of the recipient. All-*trans* RA, a derivative of Vitamin A can act as a potential T_H17 inhibitor by inducing the down-regulation of IL-6R [HILL JA ET AL 2008]. The full mechanism of RA is not understood [discussed in MUCIDA D ET AL. 2009]. Other mechanisms involved in T_{reg} versus T_H17 cell fate decision could be the influence of the TF IRF4, which is expressed in all T_H effector cells to some degree whereas it is thought to mainly play a part in the T_H2 and T_H17 differentiation program partially by inhibiting T_H1 and T_{reg} cell fate [BRUSTLE A ET AL. 2007, ZHENG Y ET AL. 2009]. $IRF4^{null}$ mutant mice have an even more severe defect in their generation of T_H17 cells than RORγt mice. It was shown that RORγt and RORα upregulation depend on IRF4 whereas STAT3 and SOCS3 are independently regulated [BRUSTLE A ET AL. 2007, HUBER M ET AL. 2008]. Enforced expression of RORγt in $IRF4^{null}$ mice leads to a partial rescue of IL-17A expression [BISWAS PA ET AL. 2009]. IRF4 also regulates the expression of IL-17A, IL-21 and IL-23R [CHEN Q ET AL. 2008, HUBER M ET AL. 2008]. A recent study suggests a direct transactivation of the *il17a* and *il21* loci by IRF4 [CHEN Q ET AL. 2008]. The role of IRF4 in T_H17 differentiation is a current focus of research and still a matter of debate [discussed in NURIEVA RI ET AL. 2008, ALTMAN A ET AL. 2008]. Most of the studies on the transcriptional regulation of the T_H17 and T_{reg} lineage have only been performed in the mouse system. It has to be addressed whether the same control elements are found in human T_H cells. One very intriguing modulatory element that has been only found in humans is a naturally occurring splice variant of FoxP3, in which the exon that is responsible for FoxP3-RORγt interaction is missing (FoxP3Δex2). How the expression of the variants is regulated is not known [ZHOU L ET AL. 2009]. The effort to understand the transcriptional rationale behind the T_H17 differentiation program acquired an interesting facet when involvement of aryl hydrocarbon receptor (AHR) was discovered. AHR is a sentinel of environmental toxins (polycyclic aromatic hydrocarbons), which acts as a ligand-dependent TF [reviewed in GU YZ ET AL. 2000]. Loss-of-function studies have suggested that IL-22 expression and to a lesser extend IL-17A & -17F expression is regulated by this TF [QUINTANA FJ ET AL. 2008, VELDHOEN M ET AL. 2008]. Besides T_H17 cells AHR can be expressed in T_{reg}, but at 50-100 fold lower level [VELDHOEN M 2009, CHANG X ET AL. 2007]. This finding poses an unequivocal link between environmental factors (e.g. pollution) and inflammation. There is some evidence that different ligands of the receptor influence the balance between T_{reg}/T_H17-commitent differently [KIMURA A ET AL. 2008]. The physiological relevance of this connection between xenobiotics and inflammation has not yet been sufficiently addressed [reviewed in ESSER C ET AL. 2009].

Stabilisation of the TH17 effector response

Once the balance is tipped and the TH17 specific transcriptional program is dominantly active, the lineage decision becomes consolidated by a chain reaction of positive feedback mechanisms. IL-6 is a strong inducer of IL-21 [SUTO A ET AL. 2008] in a RORγt independent manner [ZHOU L ET AL. 2007]. IL-21 is expressed by NKT cells and activated T cells (incl. TH1, TH2 and TH17) but not by APC. The strongest producers are follicular TH cells and TH17 cells. TH cells become sensible to IL-21 by the expression of its γc-receptor complex upon TCR signalling. IL-21 expression is further upregulated in an autocrine manner by the cytokine itself. IL-21 also acts on $CD8^+$ T cells, B cells, NK cells and DC [reviewed in LEONARD WJ ET AL. 2008]. In terms of TH17 development it has similar, possibly even redundant functions as IL-6. There is evidence that IL-21 is crucial in amplifying the precursor frequency of TH17 cells acting as an autocrine positive feedback loop [KORN T ET AL. 2007] as well as by inducing the expression of the IL-23R in a STAT3-dependent manner, which furthers the commitment to the TH17 effector response [ZHOU L ET AL. 2007, NURIEVA R ET AL. 2008]. *In vitro* loss of IL-21 decreases the number of TH17 cells. *In vivo* the situation is less clear. In most disease models in which TH17 cell are recruited, IL-21 deficiency seems to have little impact. In these instances high amounts of IL-6 seem to be able to compensate for loss of IL-21. In the absence of high levels of IL-6, IL-21 might play a more prominent role [SONDEREGGER I ET AL. 2008, ZHOU L ET AL. 2008, COQUET JM 2008]. Interestingly, in the absence of inflammation IL-21 might play a role in the maintenance of the TH17 memory pool, since it was reduced in $IL\text{-}21R^{null}$ mutant mice [KORN T ET AL. 2007].

One of the most exciting but still fairly obscure aspects of TH17 development and function is their dependence on IL-23. The most elaborate study addressing this point might have been performed by KAPLAN and colleagues in 2008 [STRITESKY GL ET AL. 2008]. They established long-term cultures of highly purified TH17 cells to test for the impact of IL-23 and lack of IL-23 also at later stages of TH17 lineage commitment. IL-23, especially in conjunction with IL-1β, maintained high long term IL-17A expression. It became apparent that IL-23 does not act on proliferation and longevity of the TH17 cells (neither the enriched $IL\text{-}17A^+$ nor the $IL\text{-}17A^-$ fraction). Most surprising, however, under the *in vitro* conditions applied, IL-23 stabilises the effector type only for the instant. As soon as the cytokine milieu is changed to either TH1 or TH2 polarising conditions *il17a*, *il17f*, *il23r*, *il22* and *rorc* transcription were diminished and a TH1- or TH2-like phenotype adopted. To date no study could show the precise role of IL-23 *in vivo*. The *in vitro* findings discussed, as well as those of other studies suggest a function in maintenance of the TH17 effector response potentially by direct signalling to the activated T cell. Notwithstanding it is clearly shown that IL-23 *in vivo* also acts in a T cell independent manner. How this pathway shapes the immune response is not well understood [UHLIG HH ET AL. 2006]. *In vivo* evidence confirming the notion that IL-23 stabilises the TH17 effector type mostly stems from loss-of-function studies where a reduction in the accumulation of TH17 cells in response to inflammatory stimuli was observed [CUA D ET AL. 2003, HARRIS TJ ET AL. 2007]. Even though the study from STRITESKY ET AL. is not sufficient to completely

change the common perception of the TH17 effector type, it poses the question whether TH17 cells meet the requirements to represent a true cell lineage which is not only temporarily but persistently distinct, comparable to TH1 and TH2 cells.

Lineage plasticity

Terminal differentiation describes an absolute state of lineage commitment of a cell or a cell clone even under conditions that direct towards a different cell fate. In the adaptive immune system lymphocytes are purposefully kept at a stage of highest flexibility in the naive state. Upon stimulation by a cognate antigen TCR, cytokine receptor and other receptor signalling are integrated within the cell into an activation pattern which elicits a specific effector response. The lineage phenotype is driven by a "master regulator" in form of a specific TF. Hinged on this TF are functions of self propagation and reciprocal inhibition. Furthermore, polarisation imprints itself onto the genetic code. These epigenetic changes lead to an individualised "memory" of a T cell or B cell clone which is passed on during clonal expansion and potentially also effects the generation of memory cells. All this together defines a true lineage.

Epigenetics

Epigenetic modifications provide a precise way of introducing heritable modifications of gene expression reflecting the "experiences" the individual cell has made. They are stable but to a variable degree plastic. Epigenetic changes can be chemical modifications of cystein-CpG or histones as well as changes in the composition of the nucleosome. The nucleosome consists of a stretch of DNA (ca. 160bp) wrapped around an octamer of histones. It is the basic structural subunit of chromatin. There are different histones that can be incorporated in a nucleosome. Epigenetic signals provide the context for the cell to be able to interpret the genetic information. They are markers that represent the chromatin structure as well as the transcriptional state of a genetic locus. There are at least three different ways employed to epigenetically regulate gene transcription: (a) Methylation status of the DNA, catalyzed by enzymes known as DNA methyl transferases (b) composition and compaction of the nucleosome and (c) post-translational histone modifications. The complex epigenetic changes of a genetic region generate a pattern that, as a whole, directs transcription in terms of allowing it (active/accessible), being inactive but poised or being silent [reviewed in WILSON CB ET AL. 2009]. DNA methylation is involved in X-chromosome inactivation in females and DNA imprinting events, which result in monoallelic gene expression. Its directive power is most influential when it is positioned at the promoter region or at a regulative element (e.g. enhancer). Cystein methylation directly blocks the binding of TF to the respective motive in the promoter region or indirectly by providing docking sites for methyl-CpG binding domain proteins (MBD). The molecular details, while well described in plants, have still been insufficiently investigated in mammalian cells. It has been reported

that certain MBDs can reverse DNA methylation in human cells [BROWN SE ET AL. 2008]. Stability of the modification over multiple cell cycles is achieved by copying the methylation pattern to the progeny by DNA methyltransferase 1. The nucleosome can be condensed to a different degree depending on the histone composition. Along with DNA methylation, the phenomenon of active DNA demethylation also exists as in the case of the IL-2 promoter which becomes demethylated within 20 minutes of stimulation.

In addition, there is a huge variety of dynamic histone modifications, such as addition or removal of methyl-, acetyl-, ubiquitin-, phosphate-, sumoyl- or ADP-ribose-groups. This can create binding sites for regulative elements that allow or impede transcription. Genetic and empirical data have been used to create a so called histone code that translates certain composition and modification patterns of histones to their functional impact. H3 and H4 are the most frequent targets of methylation. Histone methylation occurs on lysine (K) residues 4, 9, 27 and 36 on H3 and on position 20 on H4 catalysed by the histone methyltransferases. For instance, dimethylated and trimethylated H3K27 or H3K9 histones are typically found at silenced loci (heterochromatin). H3K4 di- and trimethylation is a permissive constellation whereas loci that are poised to become either active or inactive are generally free of specific histone modification or carry a bivalent pattern of inactivating and activating signals [reviewed in JANSON PC ET AL. 2009].

The difference in lineage plasticity between long-term and short-term cultures is reflected in the epigenetic changes at the genetic loci of key factors of either lineage. Only after multiple rounds of restimulation are epigenetic changes fully established to ensure long-term commitment of the cell [WEI G ET AL. 2009].

Plasticity is conserved in all TH lineages, but to different degrees

TH1 and TH2 polarised by multiple rounds of restimulation have been shown to be generally stable if subsequently cultured in the respective counter-polarising conditions [MURPHY E ET AL. 1996, reviewed in ZHU J ET AL. 2010]. Their master TF seemed to exclude each other due to reciprocal inhibition. T-bet has the potential to directly interact with GATA-3 and inhibit its binding to its DNA motifs [HWANG ES ET AL., 2005]. GATA-3 represses IL12Rβ2 and STAT4 expression [OUYANG W ET AL. 1998, USUI T ET AL. 2003]. Epigenetic changes were shown to mark the signature cytokine gene loci accordingly [CHANG S ET AL. 2007]. In short term cultures representing early TH1 or TH2 cells redirecting cell fate is easily done by changing the culture conditions. TH cell clones that had divided more than four to five times were not readily redirectable [GROGAN JL ET AL. 2001]. Upon severe force also those fully established TH1 or TH2 cells could be redirected towards the other lineage. For instance, provision of T-bet to either mouse or human TH2 cells redirected them to the expression of the TH1 cytokine IFNγ, at least in parallel to IL-4 [LAMETSCHWANDTNER G 2004]. *Vice versa*, ectopic expression of GATA3 in TH1 cells lead to IL-4 expression [LEE HJ ET AL. 2000]. In true *ex vivo* settings TH1 and TH2 cells have been mostly described as moderately plastic towards the other lineage [MESSI M ET AL. 2003].

Nevertheless, the recent appreciation of a much more diverse array of effector types in a T_H cell response led to a closer investigation on the subject of T_H effector lineage stability and the possibility of late developmental plasticity. T_{reg} cells are generally accepted to satisfy the lineage criteria. However, they seem to depend on a higher degree of transcriptional flexibility. In 2009, two groups presented convincing evidence that added a further level of heterogeneity [KOCH MA ET AL. 2009, ZHANG Y ET AL. 2009]. It was suggested that T_{reg} phenotypically "mimic" characteristic traits of the effector cell type they were suppressing. On the molecular level, this phenotypic mimicry was due to the upregulation of lineage associated TF T-bet or IRF4 upon respective polarising conditions. That way, T_{reg} seem to adapt to a specific effector lineage driven scenario enabling them to suppress efficiently in that context. But their capacity of late developmental plasticity goes beyond that. The most striking example has been reported by the group of BELKAID. In the context of lethal oral *Toxoplasma* infection, T_{reg} cells acquired a T_H1 phenotype and were shown to be proinflammatory active [OLDENHOVE G ET AL. 2009]. T_{reg} instability has been also demonstrated in EAE when T_{reg} were transferred into lymphopenic hosts [YANG XO ET AL. 2008]. In agreement, fate mapping experiments indicating a cells history of FoxP3 expression suggested that in autoimmune pathology former T_{reg} cells execute a proinflammatory, mostly T_H1-like phenotype [ZHOU X ET AL. 2009]. Those secondary proinflammatory cells mostly lost their FoxP3 expression completely. It appears as if T_{reg} with high CD25 expression (a direct transcriptional target of FoxP3) are more stable in their lineage commitment than $CD25^{low}$ T_{reg} [KOMATSU N ET AL. 2009].

Most striking in the discussion about lineages is the apparent intimate relationship between T_{reg} and T_H17 cells. As was stated before, both lines are developmentally dependent on TGFβ signalling (c.f. "Introduction: T_H17 cells"). The exposure of naive T_H cells to TGFβ results in an upregulation of FoxP3 and RORγt in parallel. Interestingly, FoxP3 engages in physical interaction with a number of TF that are involved in lineage decisions, e.g. IRF4, RUNX1, RORγt, RORα [ZHANG F ET AL. 2008, DU J ET AL. 2008, ZHENG Y ET AL. 2009]. *In vitro* restimulation of T_{reg} with IL-6 but without TGFβ induces the conversion into active T_H17 cells [XU L ET AL. 2007]. This finding might mostly reflect nT_{reg} as opposed to iT_{reg} instability since stimulation with IL-2 + TGFβ stabilises T_{reg} commitment [ZHENG SG ET AL. 2008]. Activated T_H cells that coexpress FoxP3 and RORγt non-transiently have been found in mice and humans [LOCHNER M ET AL. 2008, ZHOU L ET AL. 2008, VOO KS ET AL. 2009]. These cells can be activated to produce IL-17A.

While T_{reg} clearly are able to convert into T_H17 cells, the reciprocal event has not been described. Apart of that, T_H17 cells have proven to be highly prone to lineage instability. T_H17 cells remain instable *in vitro* even upon multiple cycles of polarisation and convert into T_H1- or T_H2-like cells [LEXBERG MH ET AL. 2008]. IL-23 has been proposed by one group, TGFβ by another to stabilise the T_H17 phenotype but only for the time of its presence [STRITESKY GL ET AL. 2008, LEE YK ET AL. 2009]. On transfer, it has been observed that T_H17

cells can turn into IFNγ producers [LEE YK ET AL. 2009]. Reported by a great number of labs, the existence of IL-17A/IFNγ double producers under inflammatory conditions has been established which introduces a "grey-zone" at least in the proposed exclusiveness of the TH1 and TH17 effector types [reviewed in ZHOU L ET AL. 2009]. Considering those and other findings on the epigenetic level, the status of the TH17 effector type is currently critically reevaluated.

Autoimmunity

The mechanisms counteracting the development of autoimmunity are recessive [RAMSDELL F ET AL. 1990, SALAÜN J ET AL. 1990] and dominant tolerance [SAKAGUCHI S ET AL. 1995]. The lack of either of them is sufficient for the spontaneous development of lethal autoimmune disease [CHATILA TA ET AL. 2000, BRUNKOW ME ET AL. 2001, ANDERSON MS ET AL. 2002]. Hence, the mere autoimmune potential is a natural part of the healthy organism. Whether this is a sign of imperfection of the immune system or the downside which comes with a physiological advantage is not understood. Only the lack of control of the autoimmune potential or an uncontrollable increase of it describes the pathophysiologic conditions that lead to autoimmunity.

Recessive tolerance is generated in the thymus during T cell development by negative selection of above-threshold TCR-binding avidities to locally presented spMHC (cf. "Introduction: Adaptive mechanisms"). Since the processes dominantly rely on the one parameter of binding avidity there are a number of possible constellations that can lead to maturation of potentially autoreactive T cells. The transcript containing the crucial epitope might not be expressed much in the relevant APCs in the thymus or might not bind well to the MHC molecules leading to a shorter half life of the complex and ultimately fewer complexes on the cell surface. On the other hand the TCR might only have weak binding potential to a spMHC. This T cells then require an exceptionally strong TCR stimulus to become primed and develop into a proinflammatory effector cell. Depending on the frequency of that potentially activating self-antigen in the periphery the cell remains naive and "ignorant" of its pathogenic potential or it receives sufficient TCR stimulation disconnected from any immunogenic context to become controlled or be deleted in the periphery.

There is genetic evidence that sustains the concept of recessive and dominant tolerance, since the defect of either has been recognised to be the cause for autoimmune diseases in humans. Strong evidence for an essential role of recessive tolerance stems from a monogenic autoimmune disease caused by the disruption of the *aire* gene, for which it was concluded that intrathymic deletion but not generation of T_{reg} cells was impaired. The human disease is

called polyendocrine autoimmune disease APECED (autoimmune polyendocrinopathy-candidiasis- ectodermal dystrophy) or APS-1 (autoimmune polyglandular syndrome type 1). Typical symptoms of the disease are mucocutaneous candidiasis, hypoparathyroidism and adrenal insufficiency. In addition, a variety of secondary symptoms can be manifested, differing from patient to patient: type 1 diabetes, reproductive organ failure, alopecia and thyroiditis. [NAGAMINE K ET AL. 1997, LISTON A ET AL. 2003, ANDERSON MS ET AL 2005]. Considering the phenotype of Aire-deficient mice and humans, negative selection in the thymus is presumably important in preventing autoimmune disease. Mutations in human FoxP3 are responsible for IPEX (immune dysregulation, polyendocrinopathy, enteropathy, X-linked) syndrome leading to a complete lack of T_{reg} function, hence eliminating dominant tolerance. Within a few years of birth these individuals develop severe autoimmune symptoms. Ultimately more than 90% of IPEX patients succumb to type 1 diabetes and ca. 70% develop thyroiditis. IBD and skin disorders are also very frequent. Some of the autoimmune disease can start as early as *in utero* (e.g. type 1 diabetis). In experimental models it was shown that these autoimmune inflammations are completely aseptic, hence the result of solely the endogenous defect [CHATILA TA ET AL. 2000]. By now at least 17 mutations at the FoxP3 locus have been described to cause IPEX [reviewed in OCHS HD ET AL. 2007]. Since autoreactive (like diabetic) T cells are present, the development of autoimmunity seems to depend on the balance between self-reactive T_H and T_{reg} cells. Every common autoimmune disease in which T_{reg} cells have been investigated display a severe distortion of T_{reg} numbers or function [OCHS HD ET AL. 2007]. Accordingly, treatment of autoimmune disease tries to influence this balance by either lowering the number of proinflammatory autoreactive T cells or by increasing the number of respective T_{reg} cells. Rapamycin, which is an inhibitor of the Akt-mTOR pathway, enhances FoxP3 transcription and renders T_{reg} resistant to apoptosis. A similar selective increase in the T_{reg} cell compartment is achieved by the administration of certain IL-2-anti-IL-2 complexes [STRAUSS L ET AL. 2007, BASU S ET AL. 2008, WEBSTER KE ET AL. 2009]. Both approaches try to achieve a dominance of a regulative T cell response to arrest the inflammatory response. Another promising approach is to try not to contradict the immunogenicity of the autoantigens, but to immunomodulate the ongoing response to selectively ablate the pathogenic part of the immune response enabling the organism to regain control of the inflammation and to return to a healthy equilibrium. To make such intervention possible, it is crucial to clearly determine the pathogenic features of an autoimmune response. This approach is the conceptional framework of this study on the pathogenicity of IL-23-induced effector cytokines in the experimental model for multiple sclerosis (MS).

Autoimmune neuro-inflammation

Multiple sclerosis

MS is a chronic inflammatory neurodegenerative disease of the central nervous system (CNS) afflicting multiple regions in brain and spinal cord. The disease mostly affects young adults with a strong bias towards females. There are clear genetic predispositions, which include differences in the HLA locus (accounts for 17-60% of the genetic susceptibility) and the cytokine receptor components IL-2Rα and IL-7Rα [HAFLER DA ET AL. 2007]. These particular genetic links have lent support to the hypothesis of a primary immunologic pathogenesis in MS [reviewed in HAUSER SL ET AL. 2006]. Common features of the disease are sensory loss, weakness in leg muscles, speech difficulties, loss of coordination and dizziness. However, the actual manifestation of the disease varies significantly between patients. MS is commonly perceived to develop in two stages. The initial phase is an adaptive immune response against the myelin sheath which is an oligodendrocyte structure surrounding the axons of neurons [BHAT R ET AL. 2009]. The loss of myelin is shortly followed by axonal degeneration (Fig. 1, left) [EVANGELIC N ET AL. 2000 & 2001]. At later

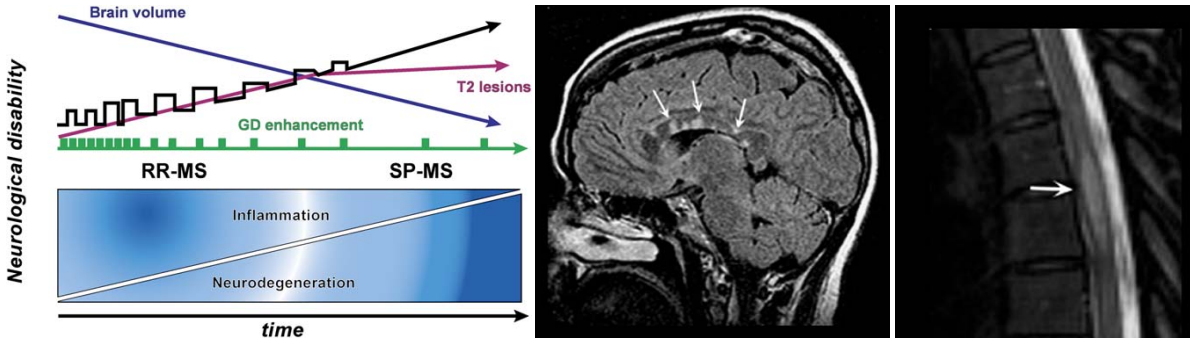

Figure 1 MS pathology. (**left**) Four clinical patterns are recognized. Approximately 85% of patients experience relapsing-remitting MS (RR-MS), characterized by the abrupt start of symptoms and acute episodes of worsening (exacerbations or relapses) with complete or partial recovery. Between these episodes, patients may be clinically stable, may experience gradual progression of disability, or may undergo a combination of both. Approximately 50% of patients with RR-MS convert to secondary progressive MS (SP-MS) within 10 years of disease onset. The secondary progressive phase is characterized by gradual progression of disability with or without superimposed relapses. In contrast, patients with primary progressive MS (PP-MS, approximately 10% of patients with MS) experience gradual progression of disability from onset without superimposed relapses. Patients with progressive relapsing MS experience gradual progression of disability from disease onset, later accompanied by one or more relapses; this clinical pattern affects ca.5% of patients. An important conceptual development in the understanding of MS pathogenesis has been the compartmentalization of the mechanistic process into two distinct but overlapping and connected phases, inflammatory and neurodegenerative. Axonal loss begins most likely at disease onset and accumulates. Conversion of relapsing-remitting to secondary progressive occurs once axon loss surpasses the capacity of the CNS to compensate for loss of function. (**middle+right**) MRI Images in MS. (**middle**) Sagittal T2-weighted fluid attenuated inversion recovery (FLAIR) image in which the high signal of CSF has been suppressed. CSF appears dark, while areas of brain edema or demyelination appear high in signal as shown here in the corpus callosum (arrows). Lesions in the anterior corpus callosum are frequent in MS and rare in vascular disease. (**right**) Sagittal T2-weighted fast spin echo image of the thoracic spine demonstrates a fusiform high-signal-intensity lesion in the mid thoracic spinal cord. [adapted from HAUSER SL ET AL. 2006].

stages the disease changes into a primarily degenerative pattern which is then not restricted to the white matter but can typically also affect the grey matter leading to a decrease in cortical thickness seen in some patients with long-standing disease [PETERSON JW ET AL. 2001]. While over time cerebral atrophy accumulates partial remyelination occurs in between the acute phase of inflammation [KORNEK B ET AL. 2000].

The aetiology of MS is still far from being established. Much of what we know of its pathophysiology we know from experimental systems, while sample analysis from MS patients grants insight directly into the human situation. MS pathogenesis implicates a T cell directed cellular immune response as a critical requirement [reviewed in SOSPEDRA M ET AL. 2005]. For instance, histopathologic analysis has revealed the presence of activated T cells in the perivascular spaces and the parenchyma in the early phase of disease. These inflammatory lesions correspond to the lesions seen in diagnostic magnetic resonance imaging (MRI). MRI is to date the most powerful standard diagnostic tool to visualise the degree and development of MS lesions in the CNS (Fig. 1, middle+right). It is feasible that MS, unlike its experimental counterpart EAE, is primarily a degenerative instead of a primarily inflammatory disease. That would imply that neuroinflammation is merely a secondary by-product of premature oligodendrocyte death. The indications in favour of this hypothesis, however, are fairly poor.

In induced autoimmune settings like most mouse model systems, priming of autoreactive T cells is enforced. How and why successful priming of naive CNS reactive T cells occurs in humans at the point of disease onset is completely unresolved. One possibility that is discussed is that T cell activation could be a result of molecular mimicry that is cross-reactivity between self-antigen and microbial epitopes. The experimental effort of testing this hypothesis is extraordinary but there is no fully conclusive evidence for it, nor any specific pathogen identified and convicted [REVIEWED IN MÜNZ C ET AL. 2009]. In favour of this concept is the finding that the frequency of T cells expressing a degenerate TCR is elevated in patients with MS and such observations are also made in experimental systems [WUCHERPFENNIG KW ET AL. 1995, LANG HL ET AL. 2002, ZHANG X ET AL. 2008]. However, not only cross-reactivity between pathogens and self could potentially mediate the brake of tolerance but also degenerate TCR recognising different self-antigens of the CNS could play a part [KRISHNAMOORTHY G ET AL. 2009].

Experimental autoimmune encephalomyelitis

EAE is a model for MS

EAE is widely studied for its close resemblance to immune infiltration and CNS pathology found in human MS [reviewed in SCHREINER B ET AL. 2009]. It can be induced by active sensitisation of experimental animals with tissue of the CNS, isolated myelin or purified

myelin proteins. The first form of induced encephalomyelitis was found in humans as a complication of the rabies vaccination designed by LOUIS PASTEUR in the late 1880s. To propagate the virus it was injected intracranially into rabbits. The spinal cord of the animals later served as the vaccination agent as it contained the rabies virus particles. The vaccination approach was generally successful but in some cases generated a certain form of ascending paralysis unrelated to the rabies virus. THOMAS M. RIVERS building on prior studies by STUART ET AL. followed the undesired consequences of vaccination with CNS homogenate up, leading (by the use of complete Freund's adjuvant) to the first description of the induction of an experimental autoimmune encephalomyelitis [KABAT EA ET AL. 1946, overview by BAXTER 2007].

The two hallmark features of MS pathology are demyelination and axonal damage. Both features are represented in EAE. The extent of axonal loss, however, depends upon the species and strain of animals and the procedure of the induction of EAE. Even though EAE can be induced in virtually any mammal, the mouse represents the most important experimental system due to the genetic and immunological tools available. EAE can be either actively induced by immunisation with a CNS restricted antigen, an antigenic peptide or otherwise passively by an adoptive transfer of encephalitogenic T cells [STROMNES IM ET AL. 2006A & 2006B]. The autoimmune reaction induced is strongly dependent on the priming of the autoreactive T cells which is biased by the chosen combination of immunogenic peptide and the MHC haplotype of the strain. The ever growing list of potential autoantigens in MS and EAE includes myelin antigens (e.g. MBP, PLP, MOG, MOBP, CNPase), glial antigens (e.g. GFAP, S100β, αβ-crystallin), and neuronal antigens (e.g. Neurofilament-L, Neurofilament-M, Contactin- 2) [SOSPEDRA M ET AL. 2005]. Most commonly used are peptides of the myelin antigens myelin basic protein (MBP), myelin oligodendrocyte glycoprotein (MOG) and proteolipid protein (PLP). In mouse EAE models with active sensitisation widespread axonal damage is found. In such lesions primary demyelination, although present, is sparse but axonal destruction with secondary demyelination prevailing. The damage in these cases is induced by autoreactive T cells [ARANAMI T ET AL. 2008]. Much more selective primary demyelination is seen in models which include demyelinating antibodies [LASSMANN H ET AL. 1983, ABOUL-ENEIN F ET AL. 2006]. Axonal injury at different stages of lesion development is still very similar to that seen in MS lesions analysed at comparable stages of their development, including some of the downstream mechanisms of axonal injury [KORNEK B ET AL., 2000]. C57BL/6 mice immunised with the MOG_{35-55} peptide (the approach used in this study) develop either self-limited monophasic or chronic EAE as compared to EAE with interchanging relapses and remissions which can be induced in SJL/J mice using PLP antigen. The latter model is in some aspects reminiscent of early human relapsing remitting MS. The most dramatic drawback of the active induction models of EAE in general is the use of a strong adjuvant that distorts the overall immune response. EAE induced by adoptive transfer of *in vitro*-activated autoimmune T cells circumvents this problem. Nonetheless, the informative value

of passive EAE is compromised greatly by the *in vitro* culture of the adoptively transferred cells and the fact that unphysiological high numbers of equally activated cells are being transferred in bulk. More recently, spontaneous EAE models have been developed with transgenic mice in which a large proportion of T cells are myelin-specific (e.g. 2D2 mice). This approach promises to circumvent the weaknesses of the active and the passive induction models of EAE but up to now no model has fully met all experimental requirements [LAFAILLE JJ ET AL. 1994, BETTELLI E 2007]. Another concern when comparing EAE and MS is the observation in many MS patients, that it is not so much the $CD4^+$ but the $CD8^+$ T cells that dominate the scene. The classical models of EAE, however, are fully $CD4^+$ T cell driven diseases which is due to the immunisation protocol [reviewed in FRIESE MA ET AL. 2005 AND 2009]. The depletion of $CD8^+$ T cell in an actively induced $CD4^+$ T cell driven EAE model results in worsening of disease severity [LINKER RA ET AL. 2005]. This suggests that tissue damage in this disease model does not rely on CTL activity. Over the time there have been attempts to install an EAE model that represents a $CD8^+$ T cell driven disease [HUSEBY ES ET AL. 2001, SUN D ET AL. 2001, FORD ET AL. 2005, JI Q ET AL. 2007, NA SY ET AL. 2008]. At least three groups have succeeded to induce a $CD8^+$ T cell driven model of EAE, which are reviewed elsewhere [WEISS HA ET AL. 2007].

In the attempt to utilise EAE to discover and develop new therapeutic targets for the treatment of MS, EAE has succeeded and failed a number of times fuelling the continual discussion about the contribution of experimental models in medical science. Without dispute, however, the value of EAE research for the establishment of basic principles of autoimmune T cell behaviour and its cross talk with the innate arm of immunity in the process of regulating CNS inflammation is unchallenged. The immunological events behind the induction of autoimmune neuro-inflammation in our model of EAE are in many parts well understood by now and thus have broadened our understanding of autoimmunity in general.

Priming of autoreactive T cells

As discussed before, tolerance is not enforced to a hundred percent during negative selection in the thymus hence autoreactive T cell do pass sometimes selection and proceed into the periphery. Tolerance is then maintained by peripheral T_{reg} [KUCHROO VK ET AL. 2002]. The possible extend of this control mechanism can be observed in transgenic T cells bearing a CNS-specific TCR; spontaneous EAE is prevented by naturally occurring T_{reg} [LAFAILLE JJ ET AL. 1994]. For autoimmunity to occur, tolerance has to be overcome. In the case of EAE this is achieved by immunising the mice in conjunction with a strong adjuvant. The T_H cells are primed in the draining lymph nodes by activated DC from the site of immunisation. This results in the activation and expansion of autoaggressive T_H effector cells. Also *in vitro* activated T cells in an adoptive transfer model cannot immediately enter the perivascular space of the CNS and induce inflammation even though they have been stimulated with their cognate antigen *in vitro*. Only *in vivo* APC contact induces the

expression of surface molecules (e.g. chemokine receptor regulation) on the activated autoreactive T cells necessary to engage and pass the blood brain barrier (BBB). It has been shown by our group that secondary lymphoid tissues (SLT) and the spleen are not necessary for this process of T cell priming, but that sufficient structural redundancy exists to allow productive ectopic APC-T cell interaction. This contrasts to B cell activation which is critically dependent on SLT [GRETCHEN ET AL. 2009]. Expanded and fully primed T cells are licensed to enter peripheral tissue to search for their cognate antigen.

Gaining entry to the CNS

The CNS, different to other peripheral organs, is to some extend sealed off against passive diffusion of molecules or chemicals as well as passive migration of cells from the systemic moiety. Even though lymphocytes from the periphery do enter in the steady-state, entry and activity of immune cells are rare and much tighter regulated than anywhere else in the body which became apparent in transplant studies [MEDAWAR PB 1948]. The CNS as well as the eyes are thus called "immuno-privileged" organs. Heavy inflammation, which can be readily initiated at any peripheral site by the induction of cell necrosis, is greatly restrained in the CNS [BELL MD ET AL. 1995]. Amongst other things there is a special tissue at the interface of the systemic circulation and the CNS that controls traffic between the two compartments which is a physical barriers of complex composition and are considerably different at different sides of the CNS (e.g. BBB and blood-cerebrospinal fluid (CSF) barrier (BCB)) (Fig. 2). Even within the so called BBB there are spatial differences in function and morphology [reviewed in BECHMANN I ET AL. 2006]. The capillary region which comprises by far the biggest area of blood-brain interface is the part that facilitates most of the solute transition. Here the BBB consists of brain microvascular endothelial cells baring an extensive expression of tight junctions surrounding the capillary lumen and counteracting passive diffusion (*zonulae occludentes*). On the other hand, an array of transporter systems and molecular pumps allow active transport into and out of the CNS. On the abluminal side of the capillary endothelium the vascular basement membrane and the basement membrane of the glia limitans (delineates the brain parenchyma) are fused. The glia limitans contains a dense network of astrocytic endfeed and some microglial endfeed. The fused basement membrane brings the endothelium into close contact to the astrocyte projections, which is essential for the generation of a *zonulae occludentes* [HAYASHI Y ET AL. 1997]. Upstream and downstream of the capillary region, the morphology of the vessel lining gradually changes. In the post-capillary regions BBB marker expression and density of tight junctions are reduced. There is a greater density of perivascular macrophages that act as scavengers that can take up molecules that might have leaked through the endothelium. They reside in a lymphatic cleft that forms at the post-capillary venules between the smooth muscle cells and pericytes that clasp around the vessel wall and the glia limitans. This additional compartment is called the perivascular space (Virchow-Robin space) and is of fundamental importance for cellular trafficking. The perivascular space drains the neuropil and is

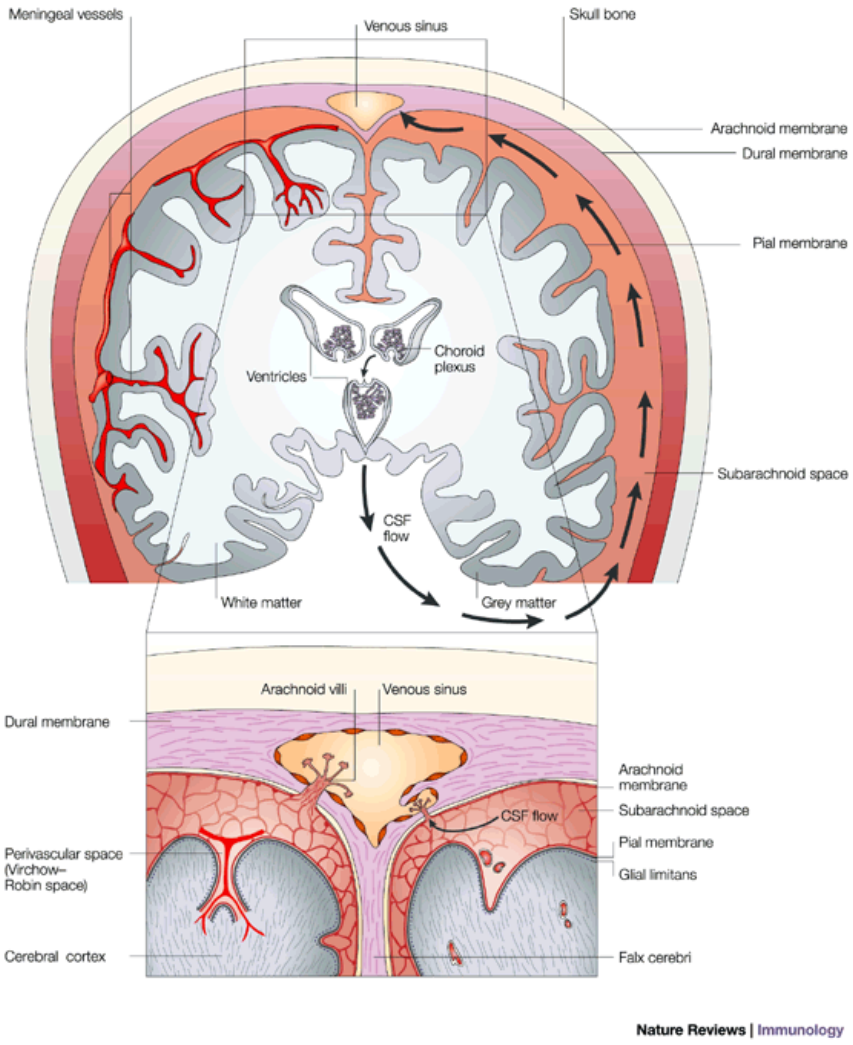

Figure 2 Anatomical structures involved in the arterial supply of the CNS and the cerebrospinal fluid circulation. The arterial supply of the brain parenchyma is derived from terminal branches of the internal carotid arteries following the brain surface in the subarachnoid space. When entering the brain parenchyma, the vessels are initially surrounded by the perivascular space, which is connected to the subarachnoid space (**insert**). Arterial supply is also provided by deep penetrating branches from the internal carotid artery. The cerebrospinal fluid (CSF) is actively secreted by the choroid-plexus epithelium that is located in the ventricular system of the brain. CSF circulates from the ventricles to the subarachnoid space located between the arachnoid and the pial membranes, and is resorbed to the systemic circulation through the arachnoid villi that extend into the venous sinuses of the cerebral hemispheres [adapted from RANSOHOFF RM ET AL. 2003].

connected to the subarachnoid space (SAS), where the CSF circulates. Hence, solutetransition and cellular diapedesis happen at different parts of the BBB and "leakage" of either is independent of the other. For cells to enter the brain they have to pass through two check points [reviewed in RANSOHOFF RM ET AL. 2003, BECHMANN I ET AL. 2006]. First through the post-capillary endothelium into the perivascular space and then from there

through the glia limitans into the neuropil (brain parenchyma) (Fig. 3). Under inflammatory conditions, like in MS or EAE, a massive influx of leukocytes across the BBB occurs inducing inflammation and tissue damage. The cellular infiltration has to be divided into perivascular and parenchymal infiltrates.

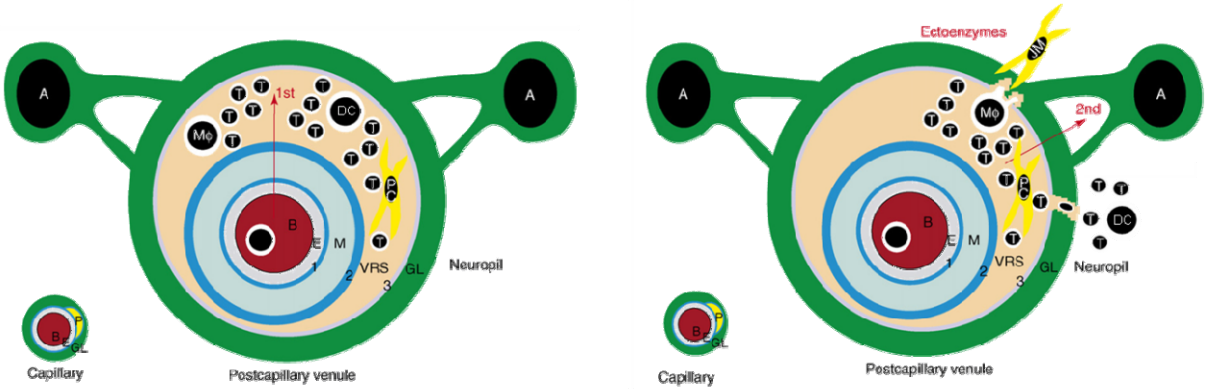

Figure 3 Two-step entry into the neuropil. (**left**) The first step in neuroinflammation. Topography of capillaries and postcapillary venules, showing a cross-section through a postcapillary venule and a capillary in the brain. Astrocytes have processes with endfeet that form the glia limitans isolating the vascular compartment (vessel wall and perivascular space) from the brain parenchyma. In postcapillary venules, three compartments with at least seven layers can be distinguished between the blood (**red**) and the neuropil: endothelium (**grey ring**), media (**light blue**), Virchow–Robin space (**orange**), glia limitans (**green**), inner and outer vascular basement membranes (**1** and **2**, **mid-blue**), and the basement membrane on top of the glia limitans (**3**, **light purple**). The different colours of the vascular and the astroglial basement membranes indicate that they differ in their content of laminin isoforms. The differences in the biochemical composition of the vascular and the glia basement membranes might explain how leukocytes under normal conditions can pass the former but not the latter. In capillaries, there is only one basement membrane (**blue**) between endothelial cells and the astrocytic endfeet of the glia limitans. This intimate contact, which is absent in venules, might drive the BBB-typical specialization of endothelium. The inner and outer vascular membranes are interconnected. The sizes of the structures have been adopted from electron microscopic analysis. The first step in neuroinflammation (1st) involves the passage of T cells and macrophages across the vascular wall and is not necessarily related to pathology. The population of perivascular cells is heterogeneous and includes leptomeningeal mesothelial cells, and macrophages which can function as antigen-presenting cells. The population of macrophages is replaced regularly by blood-borne mononuclear cells. (**right**) This step (2nd) is much more restricted and depends on the presence of 'vessel-associated' antigen-presenting cells. It is unclear whether they are located in the perivascular space or represent juxtavascular microglia (**JM**). Perivascular restimulation of T cells might be required for passage through the *glia limitans*. It is currently unknown whether the astrocytic endfeet of the glia limitans are destroyed or actively retracted during parenchymal infiltration. (Abbreviations: A, astrocyte; B, blood; E, endothelium; GL, *glia limitans*; JM, juxtavascular microglia; M, media; Mϕ, macrophage; P, pericyte; PC, perivascular cell; T, T cell; VRS, Virchow–Robin space) [adapted from BECHMANN I ET AL. 2006].

The induction of an effector response in the CNS

Only activated or memory T cells can get close enough to the CNS to perform immune surveillance or even initiate inflammation. This probably takes place in the SAS, since at the BCB sufficient adhesion molecules are constitutively expressed to allow diapedesis. CCL20 is also constitutively expressed at that site, which should attract cell bearing CCR6 on the cell surface (mostly TH17 cells) [REBOLDI A ET AL. 2009]. To start an immune response against

myelin antigen the primed autoreactive T cells have to reencounter their cognate antigen in the context of MHC II at the interface to the CNS. The oligodendrocyte, which is the target of myelin reactive T cells, cannot be directly targeted for antigen recognition because it does not express MHC II molecules. Recognition can only occur on local APC which present CNS antigen. However, costimulation is not required when the T cell has already been primed. The only APC associated with the CNS are macrophages and DC directly on the abluminal side of the blood vessel wall, mostly either in the perivasular space at the BBB or in the SAS at the BCB. Microvascular endothelial cell, although no constitutive producers of MHC II, can upregulate its expression. Nevertheless, current data does not implicate them as crucial APC in any inflammatory model [RISAU W ET AL. 1990, PRAT A ET AL. 2000, BECHER ET AL. 2000]. The first signs of inflammation in EAE have been detected in the SAS. Also a recent imaging study confirmed the SAS as the first site of T cell entry as well as APC-T cell interaction [KIVISAKK P ET AL. 2009]. Crucial for effective restimulation of the activated T cells are CD11c+ DC-like APC within the SAS and perivascular space. The myelin-reactive T cell response is shaped by the initial priming of the T cell but also by the strength of restimulation at the CNS. Dependent on that an effector response is triggered which can lead to inflammation and further recruitment of leukocytes. It can also lead to an activation of the perivascular endothelium, which starts expressing adhesion molecules and chemokines that allows further lymphocyte infiltration. As the combined inflammatory mechanisms accelerate under certain conditions infiltration of the neuropil by crossing the second physical barrier within the BBB, the glia limitans, can occur leading to deep parenchymal damage [STROMNES IM ET AL. 2008]. The cell type orchestrating this autoimmune CNS infiltration and inflammation are specifically autoreactive T_H cells. None of the aspects of disease development are completely understood, but it is the initial activation of autoreactive T cells that has received a great deal of attention in EAE research since at this priming event a crucial fate decision is made; on the one hand the potentially autoreactive T_H cells are being activated to become proinflammatory, on the other hand the T cell effector response is being predefined to allow the development of chronic autoimmune reactivity. Both aspects together are vital to completely break self-tolerance.

Role of B cells in EAE and MS

One of the typical features of MS is the presence of oligoclonal IgG. For a long time, however, the contribution of B cell in disease was completely obscure. By now clear evidence exists that B cells are present and active. Tertiary lymphoid follicles maturing antigen producing B cells can be found along the meninges of secondary progressive MS patients and the presence of these structures correlate with an adverse course of disease. At the site of neurodegeneration antibodies and complement have been found along the myelin sheath of still existing axons [reviewed in MCLAUGHLIN KA ET AL. 2008]. Most convincing of all evidence is the substantially beneficial effect of B cell depletion in patients with MS [HAUSER SL ET AL. 2008].

Experiments with B cells in EAE mostly paint a similar picture. Double transgenic mice expressing a MOG-specific TCR and the heavy chain of a MOG-specific antibody generates a highly aggressive autoimmune phenotype, most pronounced at the optical nerve and in the spinal cord [BETTELLI E ET AL. 2006, KRISHNAMOORTHY G ET AL. 2006]. More recently myelin-reactive antibodies isolated from MS patients were shown to exacerbate disease development in EAE by mediating axonal injury [MATHEY EK ET AL. 2007]. In the mouse model as well as in MS patients B cells have also been found to stimulate T_{reg} recruitment to the site of inflammation and to be able to secrete IL-10 to directly ameliorate disease [FILLATREAU S ET AL. 2002, MANN MK ET AL. 2007]. The success of the immunomodulatory drug rituximab, an antibody specific to CD20, relies at least partially on the fact that it shifts the B cell phenotype towards the regulatory one [DUDDY M ET AL. 2007].

Innate leukocyte contribution

Innate immune cells infiltrate the CNS in the course of MS and EAE development but their precise role is often not well understood. In EAE numbers can vary significantly depending on the mode of disease induction and the mouse strain used.

In MS γδT cells accumulate at the site of the lesion. γδT cells are an innate source of IL-17A, IL-17F, IL-22 and IL-21. Like in T_H17 cells, their expression is driven by RORγt and AHR. γδT cells also resemble other aspects of T_H17 cells which are connected to IL-17A expression, like IL-23R, TLR2 and dectin-1 receptor expression. They are critically protective in many mouse models of infection and chronic inflammation [reviewed in O'BRIEN R ET AL. 2010]. Tier infiltration into the CNS marks the early phase EAE development and lack of γδT cells leads to a delayed onset and less severe course of disease. The current hypothesis is that they amplify T_H17 activity, which results in a more severe inflammatory phenotype [SPAHN TW ET AL 1999, JENSEN KD ET AL. 2008, SUTTON CE ET AL. 2009, MARTIN B ET AL. 2009] contradicting studies in which γδT cell depletion by monoclonal antibodies leads to exacerbation of disease severity [PONOMAREV ED ET AL. 2005]. The use of this antibody depletion approach, however, is currently under scrutiny. It seems that the antibodies might cover but not deplete the cells as it was observed using γδT cell reporter mice [MILLER S, personal comunication]. Monocytes that enter the brain are $Ly6C^{high}$ and belong to a subset which depends on CCR2 for their trafficking. There recruitment from the bone marrow seems to be dependent on GM-CSF [KING IL ET AL. 2009]. CCR2 deficient mice exhibit less severe disease with hardly any monocyte infiltration into the CNS, although the extend of the phenotype seems to vary between labs [IZIKSON L ET AL. 2000, FIFE BT ET AL. 2000, GAUPP S ET AL. 2003, GEISSMANN F ET AL. 2003]. The monocyte migration deficit in these mice results in an increase in neutrophils and depends mostly on lack of MCP-1 signalling (CCR2 ligand) [HUANG DR ET AL. 2001]. Monocyte derived macrophages are mainly considered pathogenic, due to early depletion studies performed in EAE [BROSNAN CF ET AL. 1981]. Similarly it has been proposed that neutrophils are pathogenic in EAE [MCCOLL SF 1998]. Their migration into the CNS seem to be dependent on CXCR2, since neutrophils influx is

absent in CXCR2 deficient mice with EAE. EAE in those mice is milder which can be rescued by adoptive transfer of wild type neutrophils [CARLSON T ET AL. 2008]. Recent data from our lab suggest that neutrophil influx starts about two days before disease onset. The total amount of neutrophil invasion and the precise migration pattern into the CNS seems to greatly depend on the mouse strain [SPATH S, personal communication]. NK cells have also been depleted in mice which were then tested for their capacity to develop EAE. Depletion was conducted using anti-NK1.1 antibodies which spare NKT cells and some NK-like cells. Treatment resulted in a worsening of EAE severity suggesting a rather regulative role for conventional NK cells in EAE [ZHANG B ET AL. 1997]. None of the leukocytes mentioned above have been sufficiently studied and targeted specifically enough to form a conclusive picture on their contribution to EAE development.

IL-23 in autoimmune inflammation

The classic view on organ-specific autoimmunity

The T_H1/T_H2 paradigm provided a conceptional framework which dominantly shaped the perspective on the organisation of an adaptive immune response. Over the years evidence has accumulated that connected the presence and activity of T_H1 cells and their respective cytokines with autoimmune disease of different organs, for instance in the skin in psoriasis, in the joints in rheumatoid arthritis and in the CNS in MS/EAE. T_H2 were thought to be protective in an autoimmune setting [reviewed in GUTCHER I ET AL. 2007].

In MS patients T_H1 related markers correlated well with the course of disease activity. Elevated levels of IL-12p40 mRNA were specifically detected in the CNS of MS lesioned tissue [COMABELLA M ET AL. 1998, VAN BOXEL-DEZAIRE AH ET AL. 1999]. IL-12p40 was also found to be increased in serum and CSF of patients [DRULOVIĆ J ET AL. 1997]. Cells from these patients turned out to produce elevated levels of IL-12 (PBMNC) and were found to be more responsive to it in the CNS (elevated IL-12 receptor expression) [COMABELLA M ET AL. 1998]. Most importantly already in 1987 a clinical study has shown that administration of IFNγ leads to exacerbation of the disease [PANITCH HS ET AL. 1987]. In EAE the data was complementary. Comparing the T cell responses in different mouse strains and comparing different T_H cell effector clones in the adoptive transfer model of EAE, the conclusion always seemed to be that strains and T_H cells that were able to transfer disease carried a T_H1 bias while a T_H2 bias prevented disease [BARON JL ET AL. 1993, CONBOY IM ET AL. 1997]. In these studies IFNγ and TNFα expression clearly marked the encephalitogenic, IL-4 and IL-10 expression the non-pathogenic T_H cell response [FALCONE M ET AL. 1997]. IL-12 was recognised as the most powerful inducer of IFNγ in T_H cells and was found to be sufficient to render the relatively EAE resistant strain B10.S susceptible to disease development [SEGAL BM ET AL. 1996]. Administration of high doses of IL-12 in the otherwise monophasic EAE model in the Lewis rat induced disease relapse [SMITH T ET AL. 1997]. T cells primed *in vitro*

in the presence of IL-12 differentiated into IFNγ-producing T_H1 cells that were shown to mediate encephalitogenicity in adoptive transfer experiments. In contrast *in vitro* generated T_H2 cells failed to do so. The pivotal role of IL-12 and thus of the T_H1 effector type seemed solid as loss-of-function studies in IL-12p40^{null} mutant mice [SEGAL BM ET AL. 1998] and mice treated with neutralising anti-IL-12p40 antibody in active induction of EAE and AT experiments protected from EAE [LEONARD JP ET AL. 1995].

But at the same time when correlative evidence seemed so overwhelming a number of crucial experiments that were set to draw clear causative links between the T_H1 versus T_H2 effector cytokines and EAE disintegrated the simple paradigm rather then solidifying it. This became most apparent when gene-targeted and transgenic mouse models were used. It was surprising at first that in mice deficient in IFNγ, EAE could be induced and was even found to develop more severely as compared to wt controls [FERBER IA ET AL. 1996]. IFNγ-deficiency even converted an otherwise EAE-resistant mouse strain (Balb/c) susceptible to disease [KRAKOWSKI M ET AL. 1996]. Shortly after also TNFα had to be acquitted since loss-of-function did not lead to impairment in EAE development [FREI K ET AL. 1997]. The study of SEGAL ET AL. from 1998 which suggested an apparent crucial role of IL-12 since the p40^{null} mutant mice were completely resistant to EAE observed that this effect did not rely, as was expected, on IFNγ [SEGAL BM ET AL. 1998]. The possibility of a misinterpretation of all data on the IL-12p40 subunit became apparent when OPPMANN discovered in 2000 that p40 was actually a shared subunit of two related cytokines, IL-12 and IL-23. The prove of the mistaken identity was given by the lab of ROSTAMI as well as our lab showing that, while p40^{null} mutant mice are protected from EAE, p35^{null} mutants (lacking the second subunit of IL-12) were fully susceptible. This revelation had to change the perception on the role of the T_H1 effector type in autoimmune inflammation, since none of their signature cytokines proved guilty as mediators of encephalitogenicity. By now also IFNγR, IL-12R and IL-18 have proven their redundancy in EAE [WILLENBORG DO ET AL. 1996, CHU CQ ET AL. 2000, ZHANG GX ET AL. 2003, GUTCHER I ET AL. 2006]. The position became yet more tenuous when also the other side of the T_H1/T_H2 paradigm in autoimmunity began to weaken. While there are reports supporting the notion that IL-4 is beneficial in EAE [FALCONE M ET AL. 1997 & 1998, BETTELLI E ET AL. 1998], it was also shown that IL-4 overexpression does not attenuate disease [BETTELLI E ET AL. 1998] and in a study performed in IL-4^{null} mice on the PL/J genetic background LIBLAU ET AL. did not observe significant differences for the frequency, severity and duration of EAE and the frequency of relapses [LIBLAU ET AL. 1997]. In a more recent study on IL-4Rα-deficient mice it was shown that they develop milder disease and more pronounced remyelination [GAUPP S ET AL. 2008]. In many cases when T_H2 cells were shown to exert attenuation of EAE, apart from IL-4, IL-10 levels were also increased. Studies addressing this issue found that IL-10 mediates the gross attenuating effect while the efficacy of IL-4 in that respect is rather mild [BETTELLI E ET AL. 1998, GAUPP S ET AL. 2008]. IL-10, however, is not necessarily a T_H2 cytokine, since its secretion is regulated in all activated T_H cells independent of their effector type [reviewed in DIVEU C ET AL. 2008].

Refuge was taken in the discovery that IL-23 and not IL-12 was essential in autoimmunity. The formal prove of IL-23 being the essential cytokine in EAE was presented by CUA ET AL. in 2003 (Fig. 4) [CUA D ET AL. 2008]. In the same year the lab of ROSTAMI confirmed this on the level of the IL-12 and IL-23 receptors [ZHANG GX ET AL. 2003A & 2003B]. The particular impact connected to this finding was the description of a novel TH cell effector type, which showed IL-23 dependent expansion and expression of the proinflammatory cytokine IL-17A, TH17 cells (cf. "Introduction: T cell help in different flavours"). It is also known that polymorphisms in the IL-23 receptor are associated with a wide range of autoimmune diseases. Thus, the notion of an IL-23/IL-17A axis has emerged. The essential effect of the IL-23/TH17 pathway in autoimmunity brought TH17 initially into the spotlight and accelerated research on the topic dramatically. TH17 were acclaimed to resolve all the nagging inconsistencies in the strict TH1/TH2 paradigm, since many of the contradicting findings at least in respect to TH1 were explained by this hitherto unknown cell population.

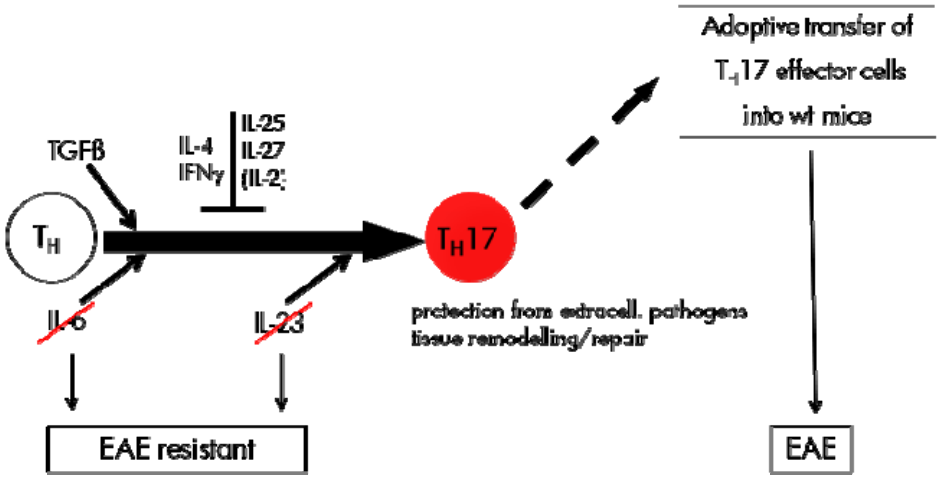

Figure 4 Development and effector functions of TH17 cells. Interference with TH17 differentiation renders mice resistant to EAE. Adoptive transfer of primed TH17 cells induces disease in the recipient mice.

IL-23, a new member to the IL-12 family of cytokines

IL-23 has been discovered in 2000 in a computational screening as a distant relative to IL-12 and IL-6. It was described as an APC-derived cytokine (like IL-12) that induces proliferation of memory T cells (unlike IL-12) [OPPMANN B ET AL. 2000]. It is a heterodimer consisting of a 19kDa 4-fold helical core subunit (p19) linked by two disulfide bonds to a 40kDa subunit (p40) which it shares with IL-12. p40 seems to be secreted as a monomer or a homodimer (function unknown), whereas p35 and p19 can be secreted only when associated with p40 [WOLF SF ET AL. 1991]. IL-23 and -12 also share a subunit in their receptor complex (Fig. 5): The heterodimeric IL-23 receptor (in this study called IL-23R) consists of the IL-23R and the IL-12β1 subunit with the former binding to the p19 and the latter to the p40 subunit of the

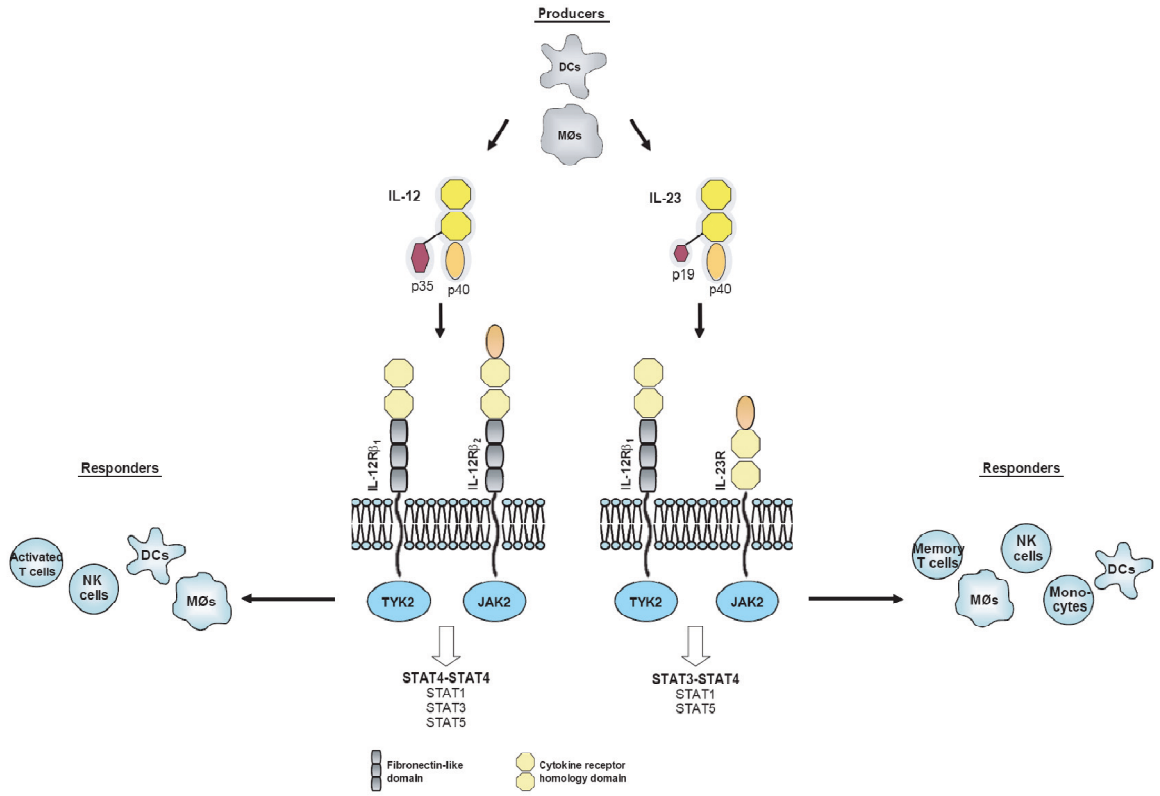

Figure 5 Sources, structure, receptors, main signalling pathways and responder cells of IL-12 and IL-23. The heterodimeric cytokines IL-12 and IL-23 – both composed of two disulfide linked subunits – are produced by APC like DC and macrophages. Despite their similarities in structure, they act on different cells of the immune system. The receptors of IL-12 and IL-23 are both composed of two chains which have to be coexpressed in order to generate functional cytokine binding sites. Following ligand binding, the signal transduction leads to activation of the JAK/STAT pathway.

cytokine. IL-23 is mainly produced by activated type I macrophages and DC from peripheral tissue. Its expression is induced by innate receptors, like TLR, whereas its signal is directed towards the innate and the adaptive arm of the immune system, as its receptor complex is expressed on DC and macrophages as well as activated T cells and NK cells [reviewed in KASTELEIN RA ET AL. 2007]. Differences in the activation pattern of their PRR influence the predominant secretion of either IL-23 or IL-12. However, whether the functional antagonism of these two cytokines is also reflected in a clear reciprocal expression pattern of two different subsets of DC is not clear. There is some evidence in favour of such a model: Gram-positive bacteria, for instance, preferentially stimulate IL-12 over IL-23 [SMITS HH ET AL. 2004]. Stimulation via G protein-coupled receptors that bind prostaglandin E2 (PGE2), ATP and other ligands are on the other hand strong differential stimulators of an IL-23 response [SCHNURR M ET AL. 2005]. This may account for the observation that pertussis toxin (PT), a virulence factor of *Bordetella pertussis*, preferentially induces IL-23 production by DC [ANDREASEN C ET AL. 2009]. PRR signalling is a complex field of synergistic effects and it is not clear to date how stimulation patterns are interpreted by the APC. Unlike most other TLR, especially when they signal in concert, TLR2 has been shown to be a poor inducer of

IL-12 in human DC. This correlates with the observation that Mycobacterium tuberculosis and the fungal antigen, zymosan, preferentially induce IL-23 [AGRAWAL S ET AL. 2003, LEIBUNDGUT-LANDMANN S ET AL. 2007, GEROSA F ET AL. 2008, ALIAHMADI E ET AL. 2009]. However, a recent study in a mouse model of pulmonary fungal infection has shown that TLR2-deficiency leads to a decrease of TH17 cells [LOURES FV ET AL. 2009]. At the point of DC-T cell interaction cytokine secretion of the DC is fine-tuned by a positive feedback signal from the T cell to the DC via cytokines, e.g. IFNγ which stimulates IL-12 production. Also via cell-cell contact there is instructive cross-talk; priming upon bacterial stimuli mostly stimulates CD40 expression on the DC, which makes them responsive to the CD40L signal from the T cell. This can lead to an enhanced IL-12 response [SCHULZ O ET AL. 2000]. Depending on the TLR context, however, this interaction can also lead to an IL-23 response [ACOSTA-RODRIGUEZ EV ET AL. 2007]. Both cytokines, IL-12 and IL-23, signal through JAK differentially activating STAT1, 3, 4 and 5. IL-23 predominantly activates STAT3 and less STAT4 which leads to hardly any STAT4 homodimerisation. IL-12 preferentially induces STAT4 activation with the outcome of mostly STAT4 homodimeres [reviewed in LYAKH L ET AL. 2008].

Two more members of the IL-12 heterodimeric cytokine family should be mentioned at that point, both were discovered as they are heterodimers of the Epstein Barr virus-induced protein 3 (EBI3), which is similar to p40, and either the p28 or the p35 subunit. IL-35, which function is largely unknown, shares the p35 subunit with IL-12 [DEVERGNE O ET AL. 1997]. There is first evidence, however, that it might a regulatory cytokine produced by T_{reg} but its possible role in autoimmunity is unresolved. [COLLISON LW ET AL. 2007]. The heterodimer of p28 combined with EBI3 can be produced by mainly myeloid cell and is termed IL-27 [PFLANZ S ET AL. 2002]. It seems to have strong immunomodulatory role mostly in respect to TH1 and TH17 development and function. Some evidence indicates a proinflammatory role in the TH1 context on the other hand it seem to be a potent inhibitor of *de novo* TH17 development [LAROUSSERIE F ET AL. 2004, EL-BEHI M ET AL. 2009]. Conversely, IL-27R deficient mice, which are reported to be hypersusceptible to EAE, produce an elevated TH17 response [BATTEN M ET AL. 2006, STUMHOFER JS ET AL. 2006]. However, IL-27 failed to suppress EAE development in an adoptive transfer model. [EL-BEHI M ET AL. 2009]. p28 can activate in an EBI3 dependent and/or independent way STAT1 and T-bet and inhibits IL-2 [KAMIYA S ET AL. 2004, OWAKI T ET AL. 2006, CRABÉ S ET AL. 2009]. The immunemodulatory role of IL-27 is very complex and still not well understood. It expands on TH1, TH2 and also CTL development and function [KAMIYA S ET AL. 2004, MORISHIMA N ET AL. 2005, REVIEWED BY KASTELEIN RA ET AL. 2007, CARL JW ET AL. 2008].

IL-23 promotes TH17 cells, the axis of evil

Once the connection between IL-23 and autoimmunity was made everything seemed to fall into place. IL-23, IL-6, IL-21 and IL-1β signalling were shown to be closely connected to TH17 development and function *in vivo*. Loss-of-function studies of all of these cytokines but IL-21

effected TH17 differentiation and, as a consequence it seemed, resulted in resistance (or nearly resistance) to EAE [EUGSTER HP ET AL. 1998, OKUDA Y ET AL. 1998, MENDEL I ET AL. 1998, SAMOILOVA EB ET AL. 1998, CUA D ET AL. 2003, SUTTON C ET AL. 2006]. In the IL-23p19-deficient mice the phenotype could be rescued by injection of IL-23 into the CNS just prior to disease onset [CUA D ET AL. 2003]. For IL-6 some studies using neutralising antibodies as compared to genetic models could not confirm the essential role of IL-6 in EAE which might be due to timing of antibody administration [SERADA S ET AL. 2008]. In the absence of IL-6 signalling EAE resistance was abrogated by depletion of T_{reg} cells which recovered the TH17 response supposedly due to a compensatory effect of IL-21 (other STAT3 signalling cytokines could also be involved) [KORN T ET AL. 2007 & 2008]. IL-21 deficient mice only showed a very mild TH17 phenotype and EAE development was unaltered [SONDEREGGER I ET AL. 2008, COQUET JM ET AL. 2008]. Nevertheless, administration of IL-21 before onset of EAE resulted in a more severe course of disease possibly due to NK cell activation [VOLLMER TL ET AL. 2005]. In all these studies the defect in TH17 cell development was presented as the most probable reason for EAE resistance but prove was missing. To show the direct connection between TH17 cells and EAE adoptive transfer model was used. *In vitro* generated TH17 *versus* TH1 cells were injected into wt mice. All of the early studies were able to show that only TH17 cells are capable of inducing severe EAE [LANGRISH CL ET AL. 2005, CHEN Y ET AL. 2006]. The obvious discrepancy that TH1 cells have served as inducers of EAE in adoptive transfer models for years was blamed on possible contamination with TH17 cell in the TH1 culture. More recent studies on adoptive transfer of TH1 and TH17 cell are in disagreement with the earlier ones (cf. "Discussion: Implications on the role of TH17 cells in autoimmunity").

Converse to the adoptive transfer studies where either TH1 or TH17 was added to the system TF-deficient mouse strains were used to address EAE development in a system lacking either effector type. STAT1-deficient mice, lacking proper TH1 differentiation, were found to be hypersusceptible, similar to IFNγ deficient mice, weaken the notion that TH1 cells are the sole mediators of encephalitogenicity [BETTELLI E ET AL. 2004]. In conflict with this T-bet-deficient mice, completely lacking IFNγ producing TH1 cells, were resistant to EAE. While this finding could be construed to strengthen the notion that TH1 cells are encephalitogenic in EAE, several reports show that T-bet-deficiency lesions not only T cell differentiation but plays a role in other developmental context [reviewed in GLIMCHER L 2007, HO IC ET AL 2009]. Comparable "master regulators" of TH17 differentiation are RORγt and STAT3. RORγt-deficiency was not sufficient to completely block EAE development but the incidence, day of onset and severity of the disease were strongly affected. It is not clear whether the ameliorated disease course is due to the reduced number of TH17 cells or due to the role of RORγt during the development of lymphoid tissues or its role in thymocyte development [SUN Z ET AL. 2000, EBERL G ET AL. 2004]. The outcome in EAE conflicts with the notion that Th17 cells are indispensible and disparate to the consequences the loss of IL-23 imposes on the development of EAE. IL-23 signals preferentially through STAT3.

Correspondingly, the absence and presence of STAT3 activation seems to have similar effects. Local STAT3 activation correlates with autoimmune inflammation in many diseases (e.g. EAE, MS, CIA, rheumatoid arthritis, systemic lupus erythematosus) [JEE Y ET AL. 2001, KRAUSE A ET AL. 2002, LIU K ET AL. 2005, FRISULLO G ET AL. 2006, HARADA T ET AL. 2007]. Loss of STAT3 in T cells renders mice resistant to EAE (the same for EAU) comparable to IL-23p19 deficiency [LIU X ET AL. 2008].

Taken together there can be no doubt that TH17 cells are present and active at the development of autoimmune inflammation. The notion that they dominate in terms of pathogenicity is currently under close investigation. TH17 cells are defined by the effector cytokines they produce. It is of interest whether these cytokine can confirm the supposed importance of TH17 cell in autoimmunity.

IL-23 induced effector cytokines

The IL-23 induced immunity plays a pivotal role in the clearance of a certain group of pathogens - possibly those that require a severe inflammatory response. In infectious models it was mostly shown that the known TH17 effector cytokines represent the functional aspects of such protective immune response. For instance, in *Klebsiella pneumoniae* infection of the lung IL-23p19-, IL-17RA- and IL-17A-deficient mice show a similar increase in susceptibility. Similar crucial involvement of IL-22 has been shown in lung infection models. The cumulative data can clearly confirm the necessity of the IL-23 induced effector cytokines for clearance of and protection from such pathogens.

In autoimmunity the situation is less clear. LANGRISH ET AL. reported in 2005 that IL-23-driven TH17 cells are the culprits in EAE. They also suggested that these effector cells are functionally distinct and that their pathogenic potential is due to the secretion of IL-17A since antibody mediated antagonisation of the cytokine *in vivo* partially ameliorates the course of EAE in SJL mice [LANGRISH CL ET AL. 2005]. IL-17A neutralisation in EAE in C57BL/6 mice by HOFSTETTER ET AL., however, only revealed a very mild effect of treatment with either monoclonal antibodies against IL-17A or with the soluble receptor of IL-17A and IL-17F [HOFSTETTER ET AL. 2005]. IL-17A deficient mice were generated by the group of IWAKURA and found to be fully susceptible to EAE after active immunisation, but demonstrate an alleviated course of clinical EAE especially in the late phase of disease. Upon adoptive transfer of TH cells, the IL-17A-deficient TH performed significantly poorer than the wt cells [KOMIYAMA Y ET AL. 2006]. These findings were then interpreted by others to represent the clear and solid proof that IL-17A is the key player in CNS autoimmune inflammation in mice and probably in men as well. Looking at the actual data that has been produced an essential role of IL-17A seems unlikely. However, the variability in the reported outcomes of the use of neutralising antibodies is surprisingly and needs clarification. Data on the role of the two other main effector cytokines of TH17 cells, IL-17F

and IL-22, in EAE development had not been reported until the time this study was undertaken (cf. "Discussion: TH17 effector function"). We hence investigated to clarify the uncertainty on the role of IL-17A and also include IL-17F and IL-22.

IL-17A and IL-17F

The IL-17 family of cytokines consists of six members: IL-17A, -17B, -17C, -17D, -17E (syn. IL-25) and IL-17F, at least three of which, IL-17A, -17E-F are produced by T cells and exert pro-inflammatory responses. IL-17B and -17C are also implicated in proinflammatory activity in autoimmune inflammation by inducing TNFα and IL-1β production but seem to be mostly expressed by non-hematopoietic cells [LI H ET AL. 2000, YAMAGUCHI Y ET AL. 2007]. The functions of IL-17B-D are otherwise ill defined. IL-17A and -17F, are signature cytokines of the TH17 response [ROUVIER E ET AL. 1993, STARNES T ET AL. 2001, KAWAGUCHI M ET AL. 2001]. The founding member of this group, IL-17A (syn. CTLA-8), was discovered in 1993 by ROUVIER ET AL. but not immediately recognised as a cytokine [ROUVIER E ET AL. 1993]. *Il17a* is highly conserved between mice (on chr. 1A4) and humans (on chr. 6p12) and also shows a high degree of homology with an open reading frame of the T lymphotrophic *herpevirus samirii* (vIL-17) [YAO Z ET AL. 1995]. IL-17F is the closest relative to IL-17A with a ca. 55% amino acid homology. The *il17f* gene is located directly juxtapositioned to *il17a* (ca. 42kb in the mouse) with inverse transcriptional orientation [reviewed in DONG C 2008]. IL-17A is secreted in a mix of both non-glycosylated and N-glycosylated forms, which migrate in SDS-PAGE at 28 kDa and 33 kDa, respectively [FOSSIEZ F ET AL. 1996]. The members of the IL-17 family have some structural homologies that define their family affiliation. They all form homodimers and contain a highly conserved part in their C-terminal region. Conserved cystein residues form a tertiary organisation, termed cystein knot, with intra- and interchain disulfide bonds [reviewed in MCDONALD NQ ET AL. 1993]. The X-ray crystallographic analysis of IL-17F illustrates the steric arrangement [HYMOWITZ SG ET AL. 2001]. A related cystein motif with six instead of four cysteins being involved but high conformational similarity is found in a group of growth factors: TGFβ, bone morphogenetic protein, platelet-derived growth factor-BB and nerve growth factor. Experience with these related factors predicted the potential for heterodimerisation [OGAWA Y ET AL. 1992]. This was later confirmed for the IL-17A and -17F subunits in mice and humans by mass spectrometry which also indicated the same anti-parallel interchain configuration as in the homodimer. Since tools for the differential analysis of the homodimer and the heterodimer of IL-17A and -17F are hardly available, there is little insight into expressional and functional differences. The few data available indicate an intermediate activity of the heterodimer relative to the homodimers, which could indicate a regulative purpose of the heterodimer [CHANG SH ET AL. 2007, WRIGHT JF ET AL. 2007, YANG XO ET AL. 2008]. Along with their structural homology, IL-17A and -17F share a noticeable degree of homology in their general cellular expression profile and in the response they elicit.

Correspondingly, the two cytokines can signal through the same receptor complex probably consisting of IL-17RA (syn. IL-17R) and IL-17RC [KUESTNER RE ET AL. 2007, WRIGHT JF ET AL. 2008]. However, binding efficiencies are different; the mouse IL-17RA subunit binds IL-17A and -17F with equal affinities while mouse IL-17RC only binds IL-17F with a relevant strength. In the human system the opposite is true [KUESTNER RE ET AL. 2007]. Mice deficient in IL-17RA or -17RC seem to be not responsive to either IL-17A or -17F in those experimental settings [TOY D ET AL 2006, ZRIOUAL S ET AL. 2008]. An IL-17RA homologue has been identified in lamprey, an ancient jawless fish, which suggest that IL-17A homologues are older than most cytokines, similar to TGFβ and IL-1. Intriguingly those three cytokines are functionally still tightly connected [KHALTURIN K ET AL. 2004]. In addition, an IL-17R homolog in zebrafish (termed SEF) has been described that functions in embryonic development [TSANG M ET AL. 2002]. Mammalian homologues of SEF were also recently identified [XIONG S ET AL. 2003, YANG RB ET AL. 2003]. IL-17RC has a possible soluble splice variant but no biological function has been reported to date [HAUDENSCHILD D ET AL. 2002, YOU Z ET AL. 2007]. Adding to the complexity of the IL-17A/IL-17F signalling is the finding that IL-17RA also partners with IL-17RB to mediate IL-17E (IL-25) signalling [RICKEL EA ET AL 2008]. IL-17E is more linked to T_H2 immune responses and in fact counteracts T_H17 differentiation [FORT MM ET AL. 2005, KLEINSCHEK MA ET AL. 2007, ZAPH C ET AL 2008]. One report in 2009 provides some loose evidence that IL-17RD associates with IL-17RA and might also be involved in IL-17A signalling [RONG Z ET AL. 2009]. The expression of the IL-17A/IL-17F receptor subunits are unfortunately not intensively studied up to now. The few publications that present data on the subject indicate that both subunits, IL-17RA and -17RC, are widely expressed in the steady-state but to some extend in a reciprocal distribution. For instance, IL-17RA seems to be highly expressed on hematopoietic cells whereas IL-17RC is rare or absent. The expression patterns also indicate a rather weak (IL-17RA) or non-existent (IL_17RC) receptor distribution in the brain but, on the other hand, noticeably high expression in the skin (and epithelial cells in general) [YAO Z ET AL. 1995, GE D ET AL. 2008, ISHIGAME H ET AL. 2009].

The two receptor subunits, IL-17RA and –RC, belong to a family of cytokine receptors with five members (IL-17RA-E). The genes encoding the subunits are clustered in mice on chromosome 6 (RA, RC, RE) and 14 (RB, RD) and in humans on chromosome 3 (RB, RC, RD, RE) [reviewed in GAFFEN SL 2009]. The extracellular moiety of IL-17RA contains fibronectin III (FNIII) like pre-ligand assembly domain that facilitates dimerisation of IL-17RA also in the absence of ligand, similar to the TNFR system. IL-17A binding is also facilitated through this domain [KRAMER JM ET AL. 2006 & 2007]. At what point IL-17RC joins the receptor complex is not known, nor is the stoichiometric composition of the signalling complex. The members of the receptor family have been identified by a computational similarity search that revealed a conserved motif in their cytoplasmatic tails, termed SEFIR domain. The SEFIR domain is critical for signal transduction and has a homologue in the Toll/Il-1R (TIR) domain which is typically involved in signalling of innate receptor systems. However, the

protein-protein binding motifs are different in the SEFIR domain resulting in the distinct downstream events [NOVATCHKOVA M ET AL. 2003]. The early downstream events of IL-17A/IL-17F signalling are not well understood. It seems obvious due to the lack of corresponding domains, that signalling does not implement the JAK-STAT pathway typically associated with cytokine signalling (even though there is weak evidence for such signalling [HUANG F ET AL. 2007]). The signalling events are more related to IL-1R, TNFR and TLR signalling, but do not rely on typical elements like MyD88, TRIF or IRAK1/4 [CHANG SH ET AL. 2006, MAITRA A ET AL. 2007]. One direct downstream element of the receptor complex is the adapter molecule ACT1 (syn. CIKS) which also contains a SEFIR domain and might bind directly to the SEFIR domain of the receptor subunits. ACT1 has a TRAF6 binding site which is also critically involved in IL-17A signalling. ACT1^{null} mutant mice proved the essential role of the adapter for the main part of the IL-17A proinflammatory program (via NF-κB), but also revealed an alternative ACT1-independent pathway since ERK1 and 2 are activated in ACT1^{null} mice in response to IL-17A [QIAN Y ET AL. 2007, LINDÉN A 2007, CLAUDIO E ET AL. 2009, reviewed in DONG C 2008]. p50 and p65 of the canonical NF-κB pathway as well as NIK of the non-canonical NF-κB pathway are involved in signalling further downstream [AWANE M ET AL. 1999, RUDDY MJ ET AL. 2004]. Some downstream elements overlap with the IL-1R and TNFR signalling pathways which might explain synergistic effects between their ligands and IL-17A signalling [IYODA M ET AL. 2010]. In parallel to NF-κB, IL-17A employs another proinflammatory pathway which leads to AP-1 via MAPK. The most important downstream effect of MAPK seems its inhibition of mRNA-destabilising proteins. Many IL-17A target chemokine mRNA are stabilised via this pathway [SHEN F ET AL. 2008, reviewed in ANDERSON P 2008]. The overall preliminary conclusion from the signalling perspective is that IL-17A/IL-17F share the same signalling elements which form a signal transduction pathway distinct from others. Nonetheless, some signalling aspects and especially the highly proinflammatory transcriptional program that is triggered, closely resemble innate receptor responses (e.g. TLR) [reviewed in GAFFEN SL 2009, HO AW ET AL. 2009]. Concerning the whole family of IL-17 receptors it is interesting that the crystal structure of IL-17RA bound to IL-17F suggests a binding motif which is conserved among all IL-17 family members. This implies IL-17RA, which is by far the subunit with the largest cytoplasmic tail, to represent a potential shared accessory subunit [ELY LK ET AL. 2009]. In favour of this hypothesis is the discovery of a functional subdomain that is unique to IL-17RA; at the carboxy-side of the SEFIR domain is a homologous structure to the BB-loop of the TIR domain (TILL) that is involved in adapter protein binding [MAITRA A ET AL. 2007, SHEN F ET AL. 2009]. This unique signalling-prone feature might be one of the functional aspects that reason the use of the IL-17RA subunit as a common receptor chain in the IL-17 receptor family.

The cellular source of IL-17A and -17F are to a great extend T_H17 cells. In EAE they are the highest producers. Other known sources are: $CD8^+$ T cells, γδ T cells, NK, NK-like cells, lymphoid tissue inducer cells (LTi) and maybe neutrophils, eosinophils and mast cells

[KAWAGUCHI M ET AL 2001, FERRETTI S ET AL. 2003, STARK MA ET AL. 2005, HUEBER AJ ET AL. 2010, reviewed in COLONNA M 2009]. Transcription of the two cytokines seems to be linked. There is emerging evidence of shared regulative genomic elements between IL-17A and -17F [AKIMZHANOV AM ET AL. 2007, WEI G ET AL. 2009]. Cells secreting either one seem to readily express the other even though not necessarily with the same kinetics. Published studies on the heterodimeric form are too premature to be conclusive [FOUSER LA ET AL. 2008]. There is only one study claiming the existence of a novel IL-17A-only producing cell type, which instead of IL-17F coproduces IFNγ [TANAKA S ET AL- 2009].

IL-17A and -17F have been implicated in a great number of physiologic and pathophysiologic conditions. The most prominent functional aspect is the induction of cytokines and other soluble immunomodulatory factors. They induce expression of TNFα, IL-1β, IL-6, GM-CSF, receptor activator of nuclear factor-κB ligand (RANKL) and prostaglandin E2 (PGE_2), which account for their proinflammatory potency [FOSSIEZ F ET AL. 1996, KOTAKE S ET AL. 1999, reviewed in KOLLS JK ET AL. 2004]. In mucosa associated tissue and the skin the secretion of antimicrobial proteins is induced, like CCL20, which is also a chemoattractant for T_H17 cells. Alongside with the induction of leukocyte recruitment IL-17A/IL-17F also induce an "opening" of the local tissue by induced secretion of matrix metalloproteinase (MMP) 3, 12 and others, depending on the tissue. In EAE this effect seems rather specific to IL-23 induced cytokines and it might allow deep infiltration of the neuropil, since MMP activity is shown to facilitate the opening of the *glia limitans*. The induction of G-CSF and ELR^+ CXC-chemokines (e.g. CXCL1/KC, CXCL8/IL-8, CXCL10/IP10) results in the activation and recruitment of neutrophils to the circulation and the site of inflammation [YE P ET AL. 2001, ZRIOUAL S ET AL. 2008, TANAKA S ET AL- 2009]. This chemokine induction is a vital part in EAE development [CARLSON T ET AL. 2008]. Neutrophil recruitment is one of the most prominent aspects of the IL-17A/IL-17F response but it is completely indirect as neutrophils do not express the IL-17 receptor complex. Neutrophils, however, do express the cytokine themselves amplifying the IL-17A driven response, as was shown in acute kidney and lung inflammation models [FERRETTI S ET AL. 2003, LI L ET AL. 2010]. In acute inflammatory conditions IL-23 induced IL-17A is often critical for disease development. The source of IL-17A in these cases is usually innate leukocytes. The main neutrophil regulating factor in general is G-CSF. Neutrophil survival, proliferation, differentiation, and function are all regulated by G-CSF. It is mainly produced by monocytes, macrophages, endothelial cells, fibroblasts and mesothelial cells, not only in response to IL-17A/IL-17F cytokines but also in response to TNFα, IL-1β, GM-CSF (*in vitro*), IL-3, IL-4 and IFNγ [NUMASAKI M ET AL. 2004, reviewed in DONG C 2008]. Early data have shown that G-CSF regulates haematopoiesis in the healthy organism as well as under inflammatory conditions. A significant role for neutrophil regulation of IL-17A has only been observed under inflammatory conditions. G-CSF does not act chemotacticly on murine neutrophils. It merely disrupts the retention signal delivered by SDF-1α in the bone marrow and thereby facilitating the migration of neutrophils across the bone marrow sinusoidal

endothelium. Neutrophils migrate then in response to the chemotactic gradient of ELR$^+$ CXC chemokines induced by IL-17A at the site of inflammation [ULICH TR ET AL. 1988, SEMERAD CL ET AL. 2002, WENGNER AM ET AL. 2008, reviewed in FURZE RC ET AL. 2008].

The former hypothesis that IL-17A/IL-17F are effector cytokines signalling to stroma but not back to lymphocytes has been weakened over the last years. The first break-through in finding a direct effect of IL-17A on lymphocytes was the reported role of IL-17A in the formation of germinal centres (GC), B cell chemoattraction and the enhancement of somatic hypermutation. MOUNTZ and colleagues observe a compelling correlative link between a higher IL-17A expression in mice prone to systemic autoimmunity, accelerated GC formation controlled by IL-17A in those mice, which leaded to critical autoantibody frequency [HSU HC ET AL. 2008]. The causal connection of IL-17A and autoantibody generation still needs to be confirmed. Nevertheless, direct signalling of IL-17A on B cells has been clearly shown [XIE S ET AL. 2010]. IL-17A involvement in B cell function has been also recently confirmed in a model of chronic gastric inflammation [ALGOOD HM ET AL. 2009]. IL-21 produced by TH17 and follicular TH cells also play a crucial role in GC development and function [KING C 2009]. Even more recently, one report by the group of FLAVELL suggested direct signalling of IL-17A to TH1 cells which was shown to inhibiting the TH1 response in an *in vivo* colitis model [O'CONNOR W JR ET AL. 2009]. Since IL-17RC has never been found to be expressed on any lymphocyte, it is reasonable to assume that IL-17A in these cases signals through receptor complex lacking the IL-17RC subunit. From a structural point of view homodimerisation of the IL-17RA subunit seems possible [ELY LK ET AL. 2009]. It is a compelling model that there different receptor complex constellations on different cell types and in different contexts leading to different signalling results. The difference of IL-17A and IL-17F responses in some contexts could also be explained by that. In some experimental setups these differences become very obvious: IL-17A plays a more pronounced role in autoimmune inflammation while IL-17F mostly neither seem to have a beneficial or pathogenic effect [YANG XO ET AL. 2008, ISHIGAME H ET AL. 2009]. In some colitis models the two brother cytokines seem to play opposite roles, IL-17A protects and IL-17F is pathogenic [OGAWA A 2004, YANG XO ET AL. 2008]. Nonetheless, the effector function can also be additive; in Il-17AR^{null} mutant mice [SCHWARZENBERGER P ET AL. 2002] as well as IL-17A/F^{null} double mutant mice [ISHIGAME H ET AL. 2009] spontaneous susceptibility to mucosal *Staphylococcus aureus* infection are common which have not been reported in the single cytokine deficient strains.

IL-22

The mouse *il22* was discovered as a gene upregulated in a T cell lymphoma upon IL-9 stimulation [DUMOUTIER L ET AL. 2000A]. Structural analysis clearly categorised it as an IL-10 cytokine family member. The human orthologue was found shortly after by two independent groups and the cytokine got its final name, IL-22. The relationship between the IL-10 family members is defined by the structure of their genomic locus, their protein

structure and their receptor use. Interestingly, the first members that joined IL-10 as a closely related molecules was a lymphocryptovirus Epstein–Barr virus (EBV) homologue which exerts almost identical bioactivity [MOORE KW ET AL. 1990]. Over the years six more viral IL-10 family members have been described all from large DNA viruses. In the mouse the cytokine family comprises seven members, in humans there are two more (IL-26, -29). They are encoded in clusters; in mice IL-10, -19, -20 and -24 are on chr. 1E4, IL-22 (NT_029419) is on chr. 10D2 and IL-28α,β (syn. IFNλ) are on chr. 7A3 (human genes [reviewed in WOLK K ET AL. 2010]). All loci share the same general intron/exon structure. There are always 5 coding exons. In the case of IL-22 they contain an open reading frame of 537bp, which encodes the 179aa IL-22 protein (In DUMOUTIER ET AL. 2000B a sixth yet untranslated exon for *il22* is described but has not been confirmed by NCBI staff). The protein is composed of antiparallel α-helices which results in a monomeric conformation of about 25kDa when glycosylated (IFNγ, in contrast, acts as a homodimer) [DUMOUTIER L ET AL. 2000A]. The tertiary structure has been confirmed by x-ray diffraction [MOORE KW ET AL. 2005, JONES BC ET AL. 2008]. It is very closely structurally related to IFNγ [WALTER MR ET AL. 1995]. Interestingly, the gene locus of IFNγ is located very close (ca. 230kb upstream) to *il22*. IL-10 family members signal through heterodimeric receptor complex of the class II cytokine receptor family (CRF2) (the same family contains the IFN receptor subunits). The extracellular moieties are composed of 2 tandem FNIII domains that bind to the ligands. The R1 chain always has the higher ligand binding specificity [LOGSDON NJ ET AL. 2002]. It carries a long intracellular tail and greatly shapes the specificity of signal transduction. To trigger intracellular signals, both R1 and R2 subunits associate with Jak family tyrosine kinases activating downstream STAT for regulation of target gene transcription [reviewed in LANGER JA ET AL. 2004]. The subfamily of IL-10 cytokine receptors contains four R1 and two R2 subunits. Not only single subunits are shared amongst the cytokines but even whole receptor complexes (the case for IL-20, -24). IL-22 signals through the IL-22R1:IL-10R2(syn. CRF2-4) (genes, respectively: *il22ra1, il10rb*) complex (in this thesis called, IL-22R). It shares its R1 subunit with IL-20 and -24, whereas its R2 subunit it shares with IL-10, IL-28α,β (and IL-26, -29 in human system). Asn54 glycosylation of IL-22 was found to be necessary for receptor binding [LOGSDON NJ ET AL. 2004]. IL-22 mostly activates STAT3 while minor phosphorylation of STAT1/5 was sometimes reported [XIE MH ET AL. 2000, NAGALAKSHMI ML ET AL. 2004, BONIFACE K ET AL. 2005, BRAND S ET AL. 2006].

Despite the high degree of similarity between the IL-10 family members and the intimate sharing of signalling elements the cytokines are distinctly different in their biological functions. IL-10, the founding member and a cytokine that is, like IL-22, expressed by activated T_H cells, is an important immunoregulatory factor (cf. "Introduction: Regulatory T_H cells: Immunity *versus* tolerance"). It acts directly on activated T cells, professional APC and NK cells which inhibits their proinflammatory program and the expression of costimulatory molecules [DEL PRETE G ET AL. 1993]. IL-22, in sharp contrast to IL-10, seems to be mostly proinflammatory. While it is also mainly produced by activated αβT cells (mostly of T_H1 and

T_H17 effector type), expression by γδT cells, $CD8^+$ T cells, NK cells, NK-like cells, monocytes and DC have been reported [WOLK K ET AL. 2006 & 2004, LIANG SC ET AL. 2006, ZHENG Y ET AL. 2007 & 2008, VOLPE E ET AL. 2008, TRIFARI S ET AL. 2009, COLONNA M 2009]. Hence, IL-22 is a shared factor of the adaptive and the innate immune system. But it does not signal back to the immune system instead mainly to stromal cells, especially epithelial cells. In the skin keratinocytes are most responsive to IL-22. In the dermis the responsiveness is much lower, due to the absence of IL-22R on melanocytes, vascular endothelium and adipocytes [WOLK K ET AL. 2006 & 2009]. Since IL-10R2 exhibits ubiquitous expression, tropism of IL-22 depends on the differential expression of IL-22R1. This restricts bioactivity of IL-22 mainly to skin, respiratory tract (lungs, trachea), digestive tract (pancreas, small intestine, liver, colon) and kidney with most expression being measured in pancreas [WOLK K ET AL. 2004]. However, it has recently been suggested by KEBIR H ET AL. that human BBB-endothelial cells might express functional IL-22R which was proposed to play a role in cellular transmigration through the BBB [KEBIR H & NINCHEN ET AL. 2007]. The main targets of IL-22 action being tissues of the body lining corresponds well with the main known functional aspects of IL-22: (a) It is a key player in the induction of antimicrobial factor expression which has strong implications in infectious disease. (b) It has a pronounced role in epidermal tissue remodelling. (c) In aseptic inflammatory conditions, for instance certain autoimmune diseases, IL-22 was shown to be significantly involved. In these often complex inflammatory disorders IL-22 activity can be protective but also pathogenic depending on the pathophysiologic context. (d) IL-22 was shown to be highly protective in hepatitis. These functional aspects will be further elaborated in the following paragraphs [reviewed in WOLK K ET AL. 2010, NINCHEN ET AL. 2010].

IL-22 can act directly on epithelial cells inducing the expression of antimicrobial proteins, like β-defensins, S100-proteins and RegIII proteins [BONIFACE K ET AL. 2005, WOLK K ET AL. 2006, ZHENG Y ET AL. 2008]. It is not surprising; therefore, that IL-22 is highly efficacious in fighting bacterial infection in the digestive tract and the lung. Hence IL-22 deficiency renders mice highly susceptible to infection models with *Citrobacter rodentium* or *Klebsiella pneumoniae* [ZHENG Y ET AL. 2008, AUJLA SJ ET AL. 2008]. The partial overlap of IL-17A/IL-22 function includes the upregulation of antimicrobial peptides and chemokine induction is observed in the *Klebsiella* model. In the gut associated *Citrobacter* infection, however, IL-17A was not crucial. The induction of IL-22 in this model was found to be IL-23 dependent, although innate cells where the crucial source of it. It is a common observation that IL-22 and/or IL-17A/F induction in infection is IL-23 dependent even if the source is not T cells. How the single cytokine expressions are coordinated and what the functional difference between the different sources has still to be explored. The role of IL-22 in fungal infection is also still open. In a model of oral candidiasis where IL-17A has been shown to play an important role the influence of IL-22 seemed to be minimal [CONTI HR ET AL. 2009]. Our lab is currently investigating on the possible role of IL-22 in disseminated *Candida albicans*

infection with primary pathogen infestation of the kidneys. Preliminary data point to a major role of IL-22, even substantially bigger than the role of IL-17A.

Especially in the skin IL-22 has a pivotal role in maintaining the tissues integrity and remodelling it under inflammatory strain. The direct effect of IL-22 on keratinocytes is the best studied IL-22/cell interaction. In keratinocytes IL-22 stimulation changes the cell differentiation program. This is due to changes in the expression pattern of genes that regulate cornification of the keratinocyte [BONIFACE K ET AL. 2005, reviewed in WOLK K ET AL. 2010]. Also cell mobility is altered by the induction of MMP1 and 3 [WOLK K ET AL. 2006]. Keratinocytes also secrete IL-20 which has very similar qualities as IL-22 and may simply amplify the IL-22 response in the epidermis. Mice overexpressing IL-20 or IL-22 are barely viable and both share a similar dramatic skin phenotype, which in many parts resembles changes in skin morphology that characterises psoriatic lesions in humans [BLUMBERG H ET AL. 2001, SA SM ET AL. 2007, WOLK K ET AL. 2009, reviewed in GRIFFITHS CE ET AL. 2007]. Many correlative studies already reported IL-22 activity in active psoriatic plaques [WOLK K ET AL. 2004, BONIFACE K ET AL. 2007, LOWES MA ET AL. 2008]. The morphological changes of the epidermal structure upon IL-22 signalling that are also typical to psoriatic lesions are: a decrease of the granular layer (hypogranulosis), increase of epidermal thickness (acanthosis) and ultimately loss of integrity of the stratum corneum [ZHENG Y ET AL. 2007, MA HL ET AL. 2008, WOLK K ET AL. 2009]. This strong influence on keratinocytes is a functional aspect that IL-22 clearly distinguishes from IL-17A/IL-17F. The latter seem to be more active in the induction of proinflammatory cytokine and chemokine secretion [NOGRALES KE ET AL. 2008].

There are a number of other inflammatory conditions in which IL-22 plays an important role in directing the local effector response - some of them autoimmune diseases. IL-22 can play a role in the pathogenesis of inflammatory bowel diseases (e.g. Crohn's disease, ulcerative colitis), as it acts directly on colonic subepithelial myofibroblasts. Similar to its role in the skin IL-22 mainly promotes the integrity of the intestinal barrier [ANDOH A ET AL. 2005]. IL-22 has also been associated with rheumatoid arthritis and its animal model collagen-induced arthritis. Its presence has been show, also the reactivity of synovial fibroblasts. Overall it seems to act similar to IL-17A as a proinflammatory factor [GEBOES L ET AL. 2009].

IL-23/IL-23R polymorphism is genetically linked with many autoimmune inflammatory conditions. Interestingly in psoriasis certain polymorphisms in the p40 and IL-23R genes promote susceptibility to psoriasis, whereas other polymorphisms in the same two genes confer protection from developing psoriasis. This is, to date still hard to interpret, but nevertheless allows the conclusion that it is important. IL-22/IL-22R genes, however, have never been connected with chronic or autoimmune inflammations, even though IL-22 is so deeply involved in inflammatory conditions like psoriatic plaque formation. Interestingly, recently ENDAM LM ET AL. reported a first link between *il22ra1* polymorphism and severe chronic rhinosinusitis [ENDAM LM ET AL. 2009].

One hallmark functional aspect on IL-22 is specific for the liver, where IL-22 induces high amounts on acute-phase proteins, like SAA, a1-anti-chymotrypsin and LPS-binding protein [DUMOUTIER L ET AL. 2000]. Beside the possible actions of these molecules there is solid evidence that IL-22 has a direct protective effect in liver injury. In many different models of induced hepatitis IL-22 deficiency lead to more severe damage, IL-22 addition decreased or prevented damage and the adoptive transfer of IL-22 producing TH17 cell were able to protect hepatocytes from cell death during the course of disease [RADAEVA S ET AL. 2004, ZENEWICZ LA ET AL. 2007]. The mechanistically underpinning how IL-22 prevents tissue injury remains unclear so far.

Taken together IL-22 as well as the other IL-23 induced effector cytokines, IL-17A and -17F, are clearly associated with inflammatory conditions in different organs. Their role seems to be to set inflammatory conditions by recruiting leukocytes and reorganising the tissue to host inflammation and allow tissue repair. This is clearly beneficial in many pathogenic conditions. However, their high proinflammatory potential and tissue remodelling licensing can also be the cause of detrimental damage to healthy tissue in cases of chronic inflammation and autoimmunity. In MS and EAE TH17 cells have arisen as possibly the one cell type mainly responsible for autoimmune lesion development. Their known weapons, which characterise their effector function, are the cytokines IL-17A, -17F and -22. Whether these molecules mediate the pathogenic potential of IL-23 and the TH17 effector type is the matter of investigation in this study.

Published Results

IL-17A AND IL-17F DO NOT CONTRIBUTE VITALLY TO AUTOIMMUNE NEURO-INFLAMMATION

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IL-22 IS EXPRESSED BY TH17 CELLS IN AN IL-23-DEPENDENT FASHION, BUT NOT REQUIRED FOR THE DEVELOPMENT OF AUTOIMMUNE ENCEPHALOMYELITIS

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IL-23-DRIVEN ENCEPHALO-TROPISM AND TH17 POLARIZATION DURING CNS-INFLAMMATION *IN VIVO*

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IL-17A and IL-17F do not contribute vitally to autoimmune neuro-inflammation in mice

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The clear association of Th17 cells with autoimmune pathogenicity implicates Th17 cytokines as critical mediators of chronic autoimmune diseases such as EAE. To study the impact of IL-17A on CNS inflammation, we generated transgenic mice in which high levels of expression of IL-17A could be initiated after Cre-mediated recombination. Although ubiquitous overexpression of IL-17A led to skin inflammation and granulocytosis, T cell–specific IL-17A overexpression did not have a perceptible impact on the development and health of the mice. In the context of EAE, neither the T cell–driven overexpression of IL-17A nor its complete loss had a major impact on the development of clinical disease. Since IL-17F may be able to compensate for the loss of IL-17A, we also generated IL-17F–deficient mice. This strain was fully susceptible to EAE and displayed unaltered emergence and expansion of autoreactive T cells during disease. To eliminate potential compensatory effects of either cytokine, we treated IL-17F–deficient mice with antagonistic monoclonal antibodies specific for IL-17A and found again only a minimal beneficial impact on disease development. We conclude therefore that both IL-17A and IL-17F, while prominently expressed by an encephalitogenic T cell population, may only marginally contribute to the development of autoimmune CNS disease.

Introduction

MS and its animal model EAE are characterized by the invasion of self-reactive Th cells into the CNS, leading to demyelination, axonal loss, and neurological impairment (1). Upon activation, Th cells can differentiate into different effector cells, depending on the makeup of the priming immune synapse as well as the cytokines present (2). For over a decade, IFN-γ–secreting Th1 cells were thought to be the pathogenic population central to the pathogenesis of autoimmunity, considering the clear association of the Th1 effector type to diseases like rheumatoid arthritis, MS, and type 1 diabetes. In their respective animal models, however, the loss of the major Th1 cytokines, IFN-γ, IL-12, and IL-18 surprisingly did not hamper disease development (3–5). In fact, IFN-γ and IL-12 deficiency led to, clinically, even more severe inflammation in collagen-induced arthritis (CIA) and EAE (3, 5, 6). After these discoveries the simplistic notion that Th1 cells and their respective cytokines are the culprits of autoimmunity had to be revised. In contrast to IL-12, its relative IL-23 was found to be essential for the development of EAE and CIA (7). The finding that IL-23 induces the expression of IL-17A by Th cells then gave rise to the notion that not Th1 but IL-17A–secreting Th cells (Th17) are the main pathogenic population in autoimmune diseases (8). This hypothesis was strongly supported by adoptive transfer models, in which the transfer of IL-17A–producing effector cells into WT hosts resulted in the initiation of autoimmunity (8, 9). The de novo lineage commitment of naive T cells toward IL-17A secretion is dependent on TGF-βR engagement (10). Additional IL-6 signaling has been identified as a costimulus, directing cell fate toward Th17 commitment. IL-17A secretion is considered the hallmark of Th17 function, as it exhibits strong proinflammatory properties (8, 11) and is widely held as being the major driving force in the pathogenesis of autoimmunity. Despite the plethora of data published on IL-17A implicating its function in physiological processes, hardly any studies reveal a true causative association (reviewed in ref. 12). Here we have generated mice in which T cells overexpress IL-17A and found that even strongly increased delivery of IL-17A by T cells into in the inflamed CNS has no impact on the pathogenesis of EAE. Also, we confirmed that *Il17a*$^{-/-}$ mice are fully susceptible to EAE. It is feasible that other Th17 cytokines can compensate for the loss of IL-17A. The closest associate to IL-17A is IL-17F (13, 14), which is encoded in a syntenic fashion and shares around 50% sequence homology and a strikingly similar pattern of expression with IL-17A (15). IL-17A/F heterodimers were described previously (16) and have been shown to signal through the same receptor complex (17, 18). In order to determine whether IL-17F contributes to the pathogenicity of Th17 cells, we generated *Il17f*$^{-/-}$ mice and explored their susceptibility to EAE. To prevent potential compensatory effects of IL-17A in *Il17f*$^{-/-}$ mice, we treated them with antagonistic anti–IL-17A mAbs. Surprisingly, we discovered even anti–IL-17A–treated *Il17f*$^{-/-}$ mice to be fully susceptible to EAE, indicating that while Th17 cells physiologically associate with an encephalitogenic state, neither IL-17A nor IL-17F appear to contribute substantially to the pathogenic function of Th17 cells in vivo.

Results

IL-17A–overexpressing T cells do not enhance the pathogenesis and clinical development of myelin oligodendrocyte glycoprotein–induced EAE. To address the impact of IL-17A expressed by CNS-invading T cells on the pathogenesis of EAE, we generated a mouse conditionally overexpressing IL-17A together with EGFP (termed

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Nonstandard abbreviations used: MOG, myelin oligodendrocyte glycoprotein.

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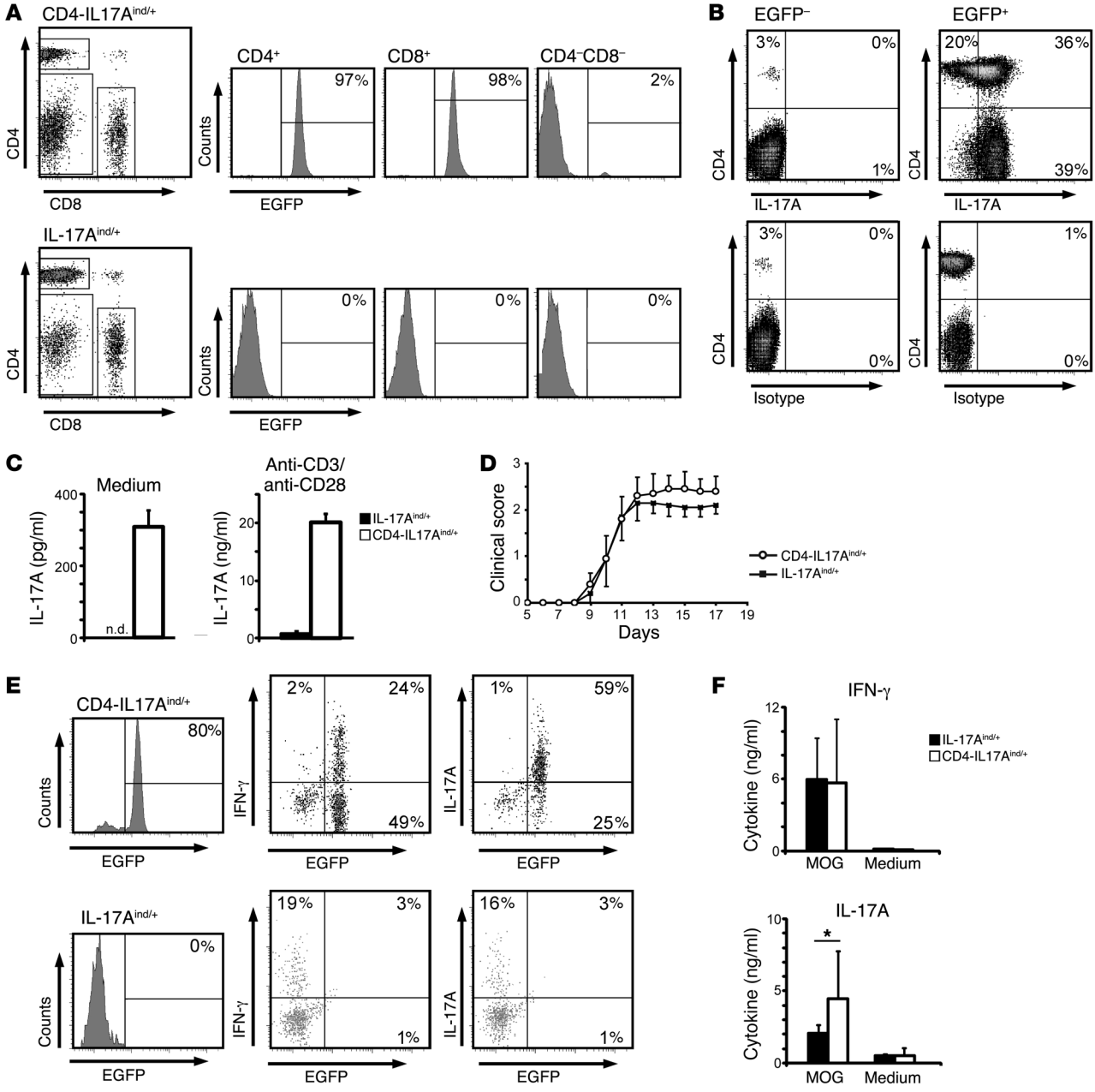

Figure 1

IL-17A overexpression does not exacerbate EAE. (**A**) LNs from CD4-IL17A$^{ind/+}$ and IL-17A$^{ind/+}$ littermates were isolated and stained for CD4 and CD8 coreceptors. EGFP expression is depicted in the histograms after indicated gating. Percentages of gated cells are shown. (**B**) Splenocytes from naive CD4-IL17A$^{ind/+}$ mice were restimulated in the presence of Brefeldin A and subsequently stained for CD4 and IL-17A. Percentages of cells in the quadrants are indicated in the corners after gating was performed on all EGFP$^-$ (plots on the left) or all EGFP$^+$ (plots on the right) cells. Data shown are representative of 3 independent experiments. (**C**) FACS-sorted CD4$^+$ T cells (2×10^5) were cultured for 24 hours in the presence or absence of anti-CD3 and anti-CD28, after which IL-17A secretion was measured by flow cytomix assay. Error bars represent mean ± SD. n.d., not detectable. (**D**) Clinical scores after $MOG_{35–55}$-induced EAE are not significantly altered by increased IL-17A expression in CD4-IL17A$^{ind/+}$ and IL-17A$^{ind/+}$ littermates. Error bars represent mean ± SEM. Data shown represent 1 out of 3 independent experiments. (**E**) Lymphocytes isolated from the diseased EAE brain and spinal cord at day 14 from CD4-IL17A$^{ind/+}$ and IL-17A$^{ind/+}$ littermates were restimulated and surface stained for CD4 and examined for EGFP expression. Further staining for IL-17A and IFN-γ was performed. Percentages of EGFP$^+$IL-17A$^+$ or EGFP$^+$IFN-γ$^+$ are given in the quadrant corners. Plots shown are gated on CD4$^+$ CNS-derived T cells. (**F**) At peak disease, mononuclear infiltrates were isolated from inflamed CNS extracts from either CD4-IL17A$^{ind/+}$ or IL-17A$^{ind/+}$ mice. Cellular extracts were cultured for 2 days in the presence of 20 μg/ml MOG peptide, after which cytokine secretions were measured by flow cytomix. Error bars represent mean ± SD and significance is shown where relevant. *P = 0.039, Student's t test.

Table 1
Detailed clinical development of EAE in IL-17A–overexpressing, IL-17A–, and IL-17F–deficient mice

	Day of onset[A]	Incidence	Maximum score[A]
CD4-IL17A$^{ind/+}$	10 ± 0.5	93% (13/14)	2.91 ± 0.29
IL-17A$^{ind/+}$	10 ± 0.3	92% (12/13)	2.67 ± 0.13
Il17a$^{-/-}$	10 ± 0.3	91% (32/35)	2.36 ± 0.13
Il17a$^{+/+}$	9 ± 0.4	100% (46/46)	2.75 ± 0.19
Il17f$^{-/-}$	11 ± 0.3	95% (37/39)	2.68 ± 0.10
Il17f$^{+/-}$	10 ± 0.3	90% (38/42)	2.74 ± 0.12
Il17f$^{+/+}$	12 ± 0.7	100% (17/17)	2.67 ± 0.15
Il17f$^{-/-}$ + αIL-17A	15 ± 0.8	75% (9/12)	2.47 ± 0.29
Il17f$^{-/-}$ + Iso. ctrl.	13 ± 0.5	83% (10/12)	2.70 ± 0.19

[A]Mean of diseased mice (Mean ± SEM). Iso. ctrl., isotype control.

IL-17A^{ind}) after excision of a loxP-flanked transcriptional STOP cassette (Supplemental Figure 1A). LN cells from IL-17A$^{ind/+}$ mice were shown to functionally express both EGFP and secrete highly upregulated levels IL-17A after in vitro Cre-mediated recombination (Supplemental Figure 1, B and C). Crossing the IL-17A^{ind} with CD4-Cre–expressing mice (termed CD4-IL17A^{ind}) generated a T cell repertoire, in which both CD4$^+$ and CD8$^+$ T cells excise the STOP cassette and express the EGFP reporter (Figure 1A). CD4-IL17A$^{ind/+}$ T cells constitutively produce IL-17A in the steady state, and this is shown to directly correlate with expression of EGFP (Figure 1B). Expression of IL-17A by T cells in CD4-IL17A$^{ind/+}$ mice did not result in an altered composition of thymus or spleen (Supplemental Table 1). Considering that IL-17A has been shown to affect granulopoiesis (19), we examined granulocyte homeostasis in the CD4-IL17A^{ind} strain. In naive mice, we observed a minor but significant increase in the number of granulocytes in peripheral blood (Supplemental Figure 1D). After immunization of CD4-IL17A$^{ind/+}$ and littermate controls with myelin oligodendrocyte glycoprotein 33–55/CFA (MOG_{35-55}/CFA), we observed a highly significant increase in serum IL-17A, which correlated to enhanced neutrophil recruitment into the spleen (Supplemental Figure 1, E and F), thus confirming in vivo the ectopic activity of IL-17A produced by CD4-IL17A$^{ind/+}$ T cells.

To verify the increased IL-17A secretion in CD4-IL17A$^{ind/+}$ T cells, we performed IL-17A–specific ELISA assays with FACS-sorted CD4-IL17A$^{ind/+}$ and IL-17A$^{ind/+}$ CD4$^+$ T cells after 24 hours in the presence or absence of CD3 and CD28 cross-linking. As expected, CD4-IL17A$^{ind/+}$ CD4$^+$ T cells constitutively secreted elevated levels of IL-17A compared with control IL17A$^{ind/+}$ T cells. This secretion was greatly enhanced after polyclonal stimulation with anti-CD3/anti-CD28 (Figure 1C). Expression of other Th-associated cytokines such as IFN-γ, IL-4, and IL-2 remained indistinguishable from control T cells in both stimulated and unstimulated cultures (data not shown). Next, we immunized CD4-IL17A$^{ind/+}$ mice with MOG_{35-55}/CFA and pertussis toxin to induce and follow progression of EAE. Surprisingly, no significant clinical differences were observed between CD4-IL17A$^{ind/+}$ and IL-17A$^{ind/+}$ littermates in a series of experiments (Figure 1D and Table 1). Consistent with the clinical disease, cellular CNS invasion was virtually identical in both groups as measured by flow cytometry (data not shown). From all CNS fractions analyzed, no significant alterations were observed with respect to IFN-γ–secreting cells. However, CNS extracts from CD4-IL17A$^{ind/+}$ mice presented with a clearly identifiable and consistent increase in IL-17A$^+$ T cells compared with IL-17A$^{ind/+}$ mice (Figure 1E). Despite similar clinical scores, flow cytometric cytokine analysis revealed a significant increase in IL-17A secretion from CNS-isolated CD4-IL17A$^{ind/+}$ T cells, while other proinflammatory cytokines associated with EAE, including IFN-γ (Figure 1F), IL-6 or GM-CSF (data not shown), remained unaltered. Taken together, exacerbated T cell–mediated delivery of IL-17A into the inflamed CNS during MOG-induced EAE does not result in an appreciable alteration of the course of disease.

IL-17A$^{ind/+}$ mice were also crossed with the *deleter-cre* strain (20). These mice (termed Del-IL17A^{ind}) showed early signs of skin inflammation and, ultimately, a developmental retardation clearly visible from P4–6 on, as can be seen in Figure 2A. This phenotype was coupled with an upregulated secretion of IL-17A by cells isolated from bone marrow, spleen, thymus, and mesenteric LNs in unstimulated cultures (Figure 2B). The IL-17A overexpression also led to a substantial increase in the number of granulocytes throughout the body, especially in bone marrow, spleen, and blood (Figure 2, C and D). Chemical analysis of the blood compartment revealed an anemia-like phenotype, consistent with granulocytosis (Table 2). We are in the process of further elucidating the clinical impact of this genotype (unpublished observations).

IL-17A function is redundant in the development of EAE. In order to improve the understanding of the role of the Th17 effector type in autoimmunity, we next analyzed the impact of loss of IL-17A on the autoreactive Th17 response in EAE. Despite the close association of IL-17A with the inflammatory milieu in EAE, we confirmed that the loss of IL-17A does not fundamentally impede the induction of the disease, which is similar to the observations made by Iwakura and colleagues (21) (Figure 3A). In the course of EAE, there was an apparent mild decrease of disease severity. Overall, the disease severity in IL-17A–deficient mice was significantly decreased (analysis of covariance, $P = 0.015$). However, the difference in the course of the disease between WT and IL-17A–deficient mice accounted for only 1.6% of the variance in the experiment, which shows how small the effect size is compared with the variance in the system (e.g., the "mouse identity" [as in residual intermouse variance] accounts for 24% of the variance). In a detailed analysis of the pooled data, we observed only a minimal, yet statistically significant (2-tailed *t* test, $P = 0.013$), difference in the day of onset of disease and an insignificant decrease in incidence and the maximal severity of disease (Table 1). The nonessential role of IL-17A in EAE development may be due to the involvement of other Th17 associated factors. To elucidate the quality of the Th17 response in the immunized IL-17A–deficient mice, we analyzed their cytokine secretion upon in vitro restimulation with MOG_{35-55} peptide, either in the absence of exogenous cytokines or under Th17 polarizing conditions (Figure 3, B and C). Surprisingly, while the IL-22 levels were similar with T cells obtained from IL-17A–deficient and WT control mice, IL-17F secretion was found to be consistently elevated in the IL-17A–deficient model. This specific increase of 1 Th17 cytokine suggests the possibility of a compensatory expression of IL-17F in the absence of IL-17A, which may contribute to disease development.

IL-17F is expressed by Th17 cells and is abundant in the inflamed CNS. A screen performed by quantitative RT-PCR of cerebelli of mice with active EAE revealed that *Il17f*, like *Il17a*, was highly expressed in the lesioned CNS as compared with cerebelli of healthy con-

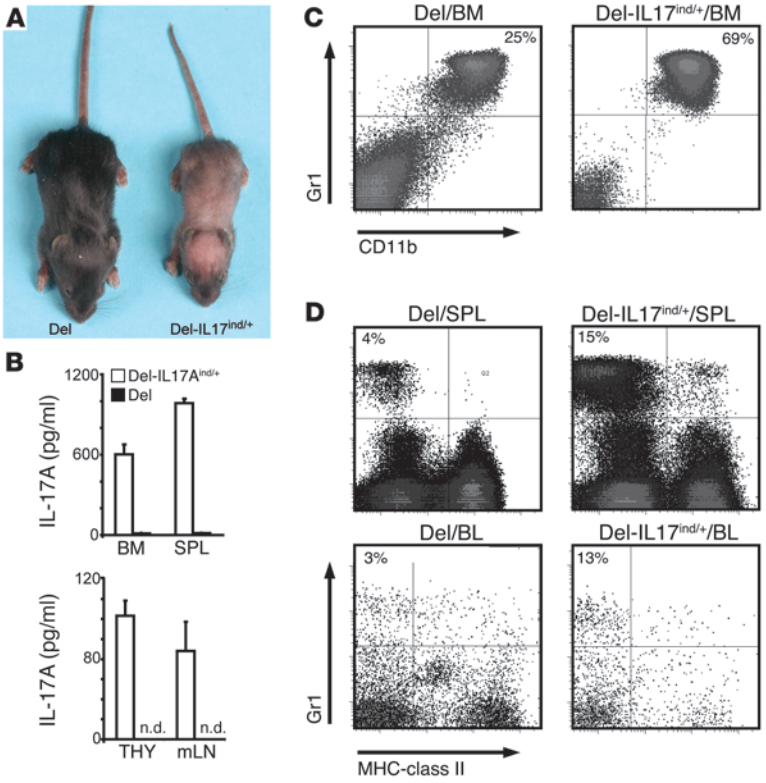

Figure 2
Systemic overexpression of IL-17A in Del-IL17A$^{ind/+}$ leads to granulocytosis and anemia. (**A**) Crossing the IL-17A^{ind} allele to the *delete-cre* strain yields mice called Del-IL17A$^{ind/+}$. Skin inflammation, stunted growth, and failure to thrive is a consistent phenotype in all mice observed when compared with *delete-cre* littermate controls (Del). (**B**) Single-cell suspensions from bone marrow, spleen (SPL), thymus (THY), and mesenteric LNs (mLN) were placed in unstimulated culture for 24 hours, after which IL-17A secretion was assayed by ELISA. Error bars represent mean ± SEM. (**C**) Bone marrow cells were isolated from Del-IL17A$^{ind/+}$ mice and littermate controls and surface stained for Gr1 and CD11b. Percentages of gated granulocytes are shown in quadrant corners. (**D**) Isolated spleen cells and PBMCs from the indicated genotypes were stained for MHC-class II and Gr1. Percentages of gated cells are shown. (**A**–**D**) Data shown are representative of at least 2 independent experiments.

trols (Figure 3D). To assess the source of IL-17F in the context of EAE, we restimulated in vivo primed splenocytes in vitro with MOG_{35-55} under Th17 polarizing conditions and subsequently analyzed the cytokine profile by intracellular cytofluorometric analysis. IL-17F expression was restricted to MOG-responsive and potentially encephalitogenic CD4$^+$ Th cells that are also producing IL-17A (Figure 3E).

The observation that both cytokines are found in the inflamed CNS and mark those highly pathogenic Th17 cells that have been associated with autoreactive lesions suggests that IL-17F is an encephalitogenic cytokine with functional relevance in CNS autoimmune inflammation.

Generation and analysis of the IL-17F–deficient mice. To ultimately determine whether IL-17F contributes to the development of EAE, we generated IL-17F–deficient mice by the replacement of exons 2 and 3 with a lacZ reporter cassette. IL-17F deficiency was confirmed by ELISA (Supplemental Figure 1G). Homozygous offspring were viable and showed neither developmental malformation nor any evident immunodeficiency under SPF conditions. There were no apparent alterations in the cellular composition of the immune system in homeostasis as shown by FACS analysis for spleen and thymus in Supplemental Table 2.

Up to 42 mice per group (+/+, +/−, −/−) in 6 independent experiments were immunized with MOG_{35-55}/CFA, and the clinical development of EAE was monitored daily. In line with the report by Dong and colleagues (22), the lack of IL-17F did not have any visible consequences on clinical EAE development as shown in Figure 4A. *Il17f*$^{-/-}$ mice did not show any alteration in the day of disease onset, maximum score, or incidence when compared with IL-17F competent (+/+, +/−) mice (Table 1). In accordance

Table 2
Chemical analysis of the blood compartment

Blood	Del-IL17A$^{ind/+}$ [A]			Del[B]		
wbc (×10^3 cells/μl)	4.20	1.77	1.35	1.53	1.50	3.63
rbc (×10^6 cells/μl)	6.18	5.34	3.45	4.36	8.74	7.98
Hemoglobin (g/dl)[C]	9.00	7.80	8.00	14.20	14.60	12.60
Hematocrit (%)[D]	31.20	27.30	29.50	46.00	47.20	44.80
Mean corpuscular volume (fl)[E]	50.40	50.90	46.10	53.90	54.00	56.00
Mean corpuscular hemoglobin (pg)[F]	14.50	14.60	12.50	16.20	16.70	15.70
Mean corpuscular hemoglobin concentration (g/dl)	28.70	28.70	27.00	30.00	30.90	28.00

[A]IL-17A$^{ind/+}$ mice crossed with the *deleter-cre* strain. [B]*deleter-cre* control littermates. [C]$P = 0.0015$. [D]$P = 0.0002$. [E]$P = 0.0301$. [F]$P = 0.0347$. Routine CBC analysis was performed on blood samples from the indicated mice ($n = 3$). Each column represents an individual mouse. Significant alterations in values are shown. Data shown are representative of at least 2 independent experiments.

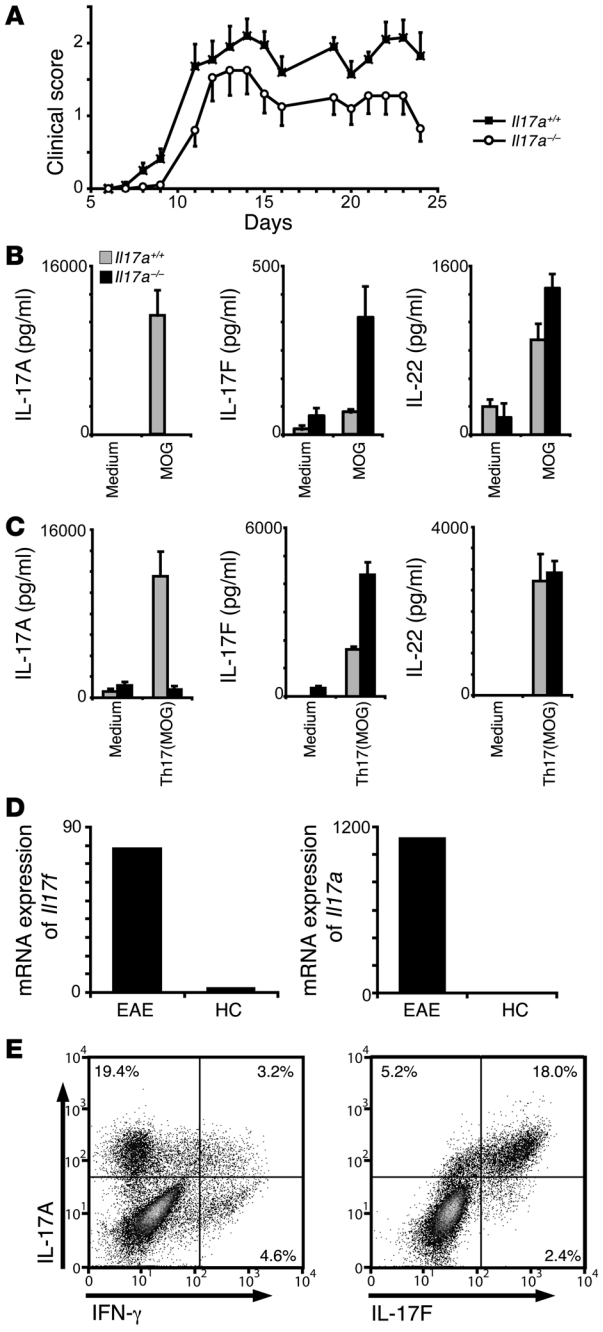

Figure 3
IL-17A is redundant in the induction of EAE, which could be due to a compensatory increase of IL-17F production. (**A**) EAE was induced in *Il17a*$^{-/-}$ and *Il17a*$^{+/+}$ mice by immunization with $MOG_{35–55}$/CFA. The graph shows the development of EAE according to clinical scores (n = 10; SEM as indicated) in 1 out of 2 independent experiments. (**B** and **C**) Th17-cytokine profile measured by ELISA of splenocytes isolated from mice with active EAE and restimulated with $MOG_{35–55}$ with (**C**) or without (**B**) the addition of Th17 polarizing conditions for 2 days. Error bars represent mean ± SEM. (**D**) Comparative mRNA expression analysis of *Il17f* and *Il17a* in the cerebellum of mice at peak EAE versus healthy controls (HC). The data represent 1 of 2 independent experiments (n = 4). (**E**) Th17 cells were generated in vitro from $MOG_{35–55}$-immunized C57BL/6 mice. Splenocytes were harvested 7 days after immunization, Th17 polarized, and analyzed by intracellular cytokine staining for IL-17A, IL-17F, and IFN-γ. Percentages of gated cells are shown. A representative of 3 independent experiments is shown.

with clinical EAE scores, histological analysis of spinal cord cross sections displayed no discernable features among WT mice and heterozygous *Il17f*$^{+/-}$ and homozygous *Il17f*$^{-/-}$ mice. Inflammation caused a severe impairment of myelinated and axonal structures, subsequently inducing reactive astrogliosis (Figure 4B). Detailed analysis of CNS-infiltrating cells by flow cytometry revealed no change in cell numbers or makeup of CNS-invading leukocytes (Figure 4C).

We did not observe any relevant difference in the capacity of *Il17f*$^{-/-}$ mice to initiate CD4$^+$ T cell priming and the effector T cell response. *Il17f*$^{-/-}$ and control mice were immunized against $MOG_{35–55}$, and primed lymphocytes were isolated from draining LNs prior to disease onset (Figure 5, A–C) and were subsequently challenged in vitro with their cognate antigen. Lymphocytes from all groups responded with the same degree of proliferation (Figure 5C). The full susceptibility of *Il17f*$^{-/-}$ mice to EAE could have resulted from a compensatory increase in the production of IL-17A. However, *Il17f*$^{-/-}$ lymphocytes obtained from the spleen showed a consistent decrease in the production of IL-17A (Figure 5A) and the frequency (Figure 5B) of IL-17A–secreting cells. Even upon overwhelming in vitro polarization toward the Th17 lineage, the proportion of IL-17A–producing T cells and the overall amount of secreted IL-17A decreased in IL-17F–targeted cells compared with WT cells under our in vitro conditions (Figure 5D). While our data indicate some degree of haploinsufficiency of the IL-17F allele (Figure 5E and Supplemental Figure 1H), targeting this locus had an inhibitory impact on IL-17A expression in vitro. We next addressed whether CNS-invading Th17 cells of the *Il17f*$^{-/-}$ mice express altered levels of IL-17A, but, in contrast to the in vitro results, we found no significant difference (Figure 5F).

Neutralization of IL-17A in IL-17F–deficient mice does not diminish EAE. Given the shared receptor specificities of IL-17F in IL-17A, the possibility exists that one cytokine is able to compensate for the loss of the other in vivo. Therefore, without removing both cytokines from EAE disease progression, redundancy of either IL-17A or IL-17F is open to interpretation. To remove both IL-17A and IL-17F during the course of EAE, we treated IL-17F–deficient mice twice a week with 200 μg of an antagonistic anti–IL-17A mAb (9). Upon repeated treatment of WT and *Il17f*$^{-/-}$ mice with either anti–IL-17A or rat-IgG1 isotype control, we could verify the titer (approximately 125 μg/ml) of the agonist in peripheral blood. The blocking capacity of the antibody found in those mice compared with the neutralizing antibody titrated directly on an IL-17A protein standard (Supplemental Figure 2A). To further control for the capacity of anti–IL-17A mAbs to block IL-17A, we treated immunized CD4-IL17$^{ind/+}$ mice with 100 μg of anti–IL-17A and found that even the accumulation of serum IL-17A in the overexpressing mice could be completely abolished (Supplemental Figure 2B). Thus, our in vivo neutralization protocol was confirmed to be an appropriate method to induce IL-17A deficiency in our mouse models. To implement this system in our clinical investigations, we treated *Il17f*$^{-/-}$ mice with anti–IL-17A mAbs. We discovered a slight trend toward disease amelioration (Figure 6), which matches our findings using *Il17a*$^{-/-}$ mice and the observations by Hofstetter et al. (23). While blockade of IL-17A has some beneficial effect, we

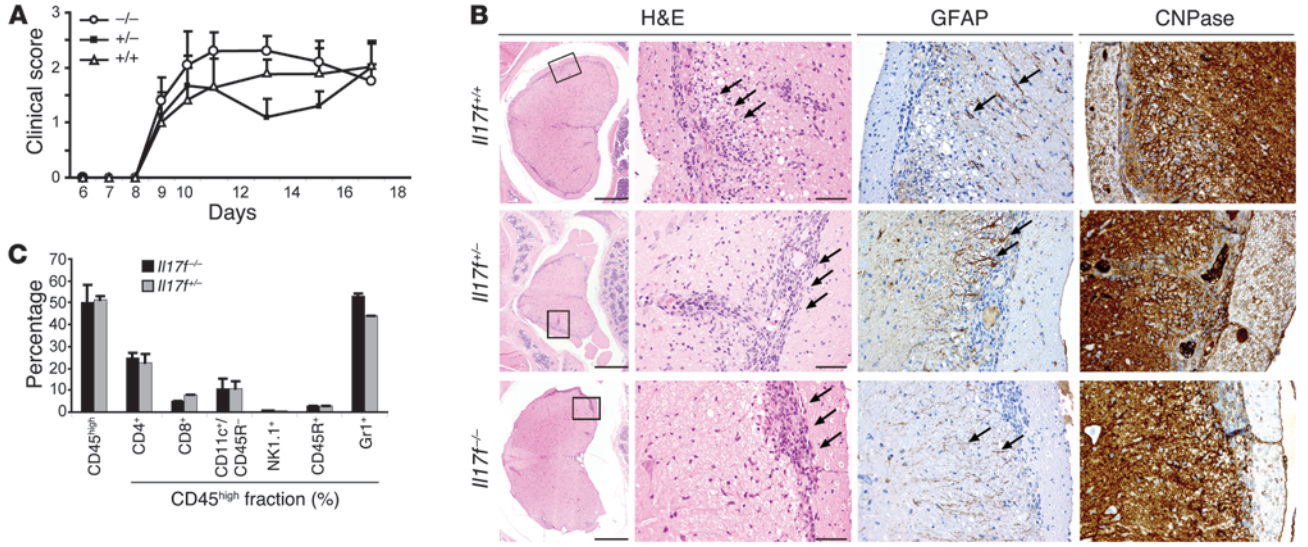

Figure 4
IL-17F is not required for the development of EAE. (**A**) EAE was induced in *Il17f*$^{-/-}$, *Il17f*$^{-/+}$, and *Il17f*$^{+/+}$ mice by immunization with $MOG_{35–55}$/CFA. The graph shows the development of EAE according to clinical scores in 1 out of 5 independent experiments. Error bars represent mean ± SEM. (**B**) Spinal cord cross sections, in accordance with clinical EAE scores, displayed similar inflammatory lesions (H&E staining; arrows). Inflammation caused an impairment of myelinated structures (staining for CNPase) and induced a reactive astrogliosis (staining for glial fibrillary acidic protein [GFAP]). All stainings were performed on serial sections. Scale bars: 500 μm (first column); 50 μm (second, third, and fourth columns). Rectangle in first column represents the area shown in second, third, and fourth columns. (**C**) Detailed analysis of infiltrating lymphocytes into cerebellum and spinal cord was performed by cytofluorometric analysis of surface marker staining. $CD45^{high}$ cells represent the CNS-invading leukocytes, which were gated on for detailed analysis. Error bars represent mean ± SEM.

conclude that IL-17A and IL-17F, alone or in tandem, do not contribute critically to the development or progression of EAE.

Discussion

After the discovery of distinct T cell polarization patterns by Mosmann and colleagues (24), it was accepted for more than a decade that IFN-γ–secreting Th1 cells are the main encephalitogenic population in CNS inflammatory diseases such as MS and EAE. In addition, Th1-promoting factors such as IL-12 and IL-18 were considered indispensable for the initiation of autoimmune disease in mice. This simplistic paradigm had to be revised when it was discovered that mice deficient in IFN-γ, TNF-α, IL-12p35, and IL-18 are either fully susceptible to EAE or hypersusceptible (3–5, 25). While IL-12 is dispensable for the induction of EAE, its close relative IL-23 has been demonstrated to be absolutely essential. Langrish et al. initially were able to show that IL-23 induces the secretion of IL-17A by effector T cells (9). IL-17A–secreting effector T cells were ultimately termed Th17 cells and are now established as a distinct helper T cell subset. In addition, the close association of Th17 cells with inflammatory autoimmune diseases such as rheumatoid arthritis, MS, and psoriasis has clearly marked this population as pathogenic (8). Several reports have elucidated the conditions to polarize toward this lineage in vitro (8, 10). While IL-17A is now considered to be the main driving force behind tissue inflammation, to this day, virtually all claims are based on a correlative relationship between Th17 cells and their presence in an inflammatory lesion. Assuming that IL-17A drives the inflammatory process and could contribute to blood brain barrier breakdown (26) and increased neutrophil activity (19), we generated transgenic mice, in which T cells produce high levels of IL-17A. Surprisingly, however, greatly increased levels of T cell–derived IL-17A expression did not impact on the development of EAE or on the quality and quantity of inflammation in the CNS. Hofstetter et al. were the first to block IL-17A in EAE and found only a minimal efficacious effect in vivo, regardless of whether they blocked IL-17A alone with an antagonistic mAb or IL-17A and IL-17F combined using a soluble IL-17 receptor (23). Komiyama et al. previously addressed the role of IL-17A in EAE by generating a deficient mouse strain (21). Unlike the deficiencies in IL-23 and IL-6, which render mice completely resistant to EAE, loss of IL-17A does not prevent disease development. However, IL-17A–deficient mice display a significantly less severe disease at late time points (26 days after immunization) and a major loss of encephalitogenic capacity after adoptive transfer of in vitro expanded T cells. However, the cells were kept in culture for 4 days and the impact on Th polarization was not evaluated. Therapeutic targeting of IL-17 using a vaccination strategy showed a more robust efficacy but did not lead to complete resistance to EAE (27). The far more critical role of IL-23 and IL-6 in the development of autoimmune inflammation indicates that the associated disruption of IL-17A and IL-17F production is a symptom but not the main cause of the complete EAE resistance observed in IL-23– and IL-6–deficient mice. Matching our conclusions, in other models of autoimmunity such as experimental autoimmune uveitis (EAU), the function of IL-17A appears to be redundant (28). Luger et al. recently demonstrated that while IL-17 can participate in the pathogenesis of EAU, it has by no means an essential role (29). The concept that IL-17A itself is not likely the only pathogenic molecule generated by Th17 cells was further supported by the report of McGeachy et al., who could demonstrate that IL-23–driven Th cells but not TGF-β/

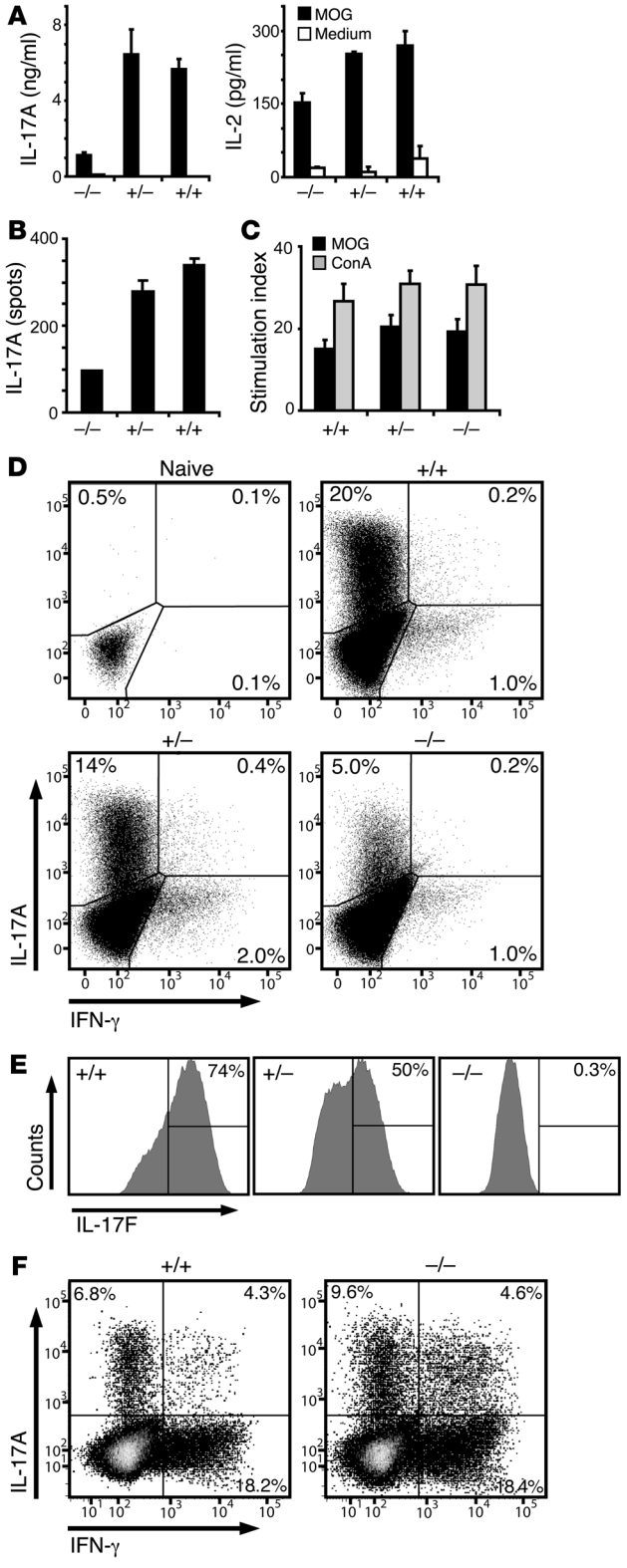

Figure 5
Loss of IL-17F does not impact on T cell priming and does not lead to compensatory upregulation of IL-17A. (**A**–**C**) Mice were immunized with $MOG_{35–55}$/CFA and lymphocytes were isolated from LNs prior to disease onset at 7 days after immunization. Cells were rechallenged with 50 μg/ml of $MOG_{35–55}$, and IL-17A and IL-2 were measured by ELISA (**A**) and ELISPOT (**B**). (**C**) Proliferation of effector Th cells upon stimulation with $MOG_{35–55}$ peptide or concanavalin A (ConA) was measured by thymidine incorporation. (**A**–**C**) A representative of 3 independent experiments is shown. Error bars indicate SEM of measured replicates. (**D**) Splenocytes from naive mice were polarized toward the Th17 effector type in vitro, and IL-17A, IL-17F, and IFN-γ were measured by intracellular cytokine staining. Dot plots are gated on Th cells ($CD4^+$), and histograms are gated on Th17 cells ($CD4^+$, $IL\text{-}17A^+$). (**E**) Within the IL-17A–expressing Th17 compartment, IL-17F production is shown for each genotype. (**F**) CNS-infiltrating lymphocytes were isolated on day 24, after immunization from severely sick mice, restimulated with PMA/ionomycin and Brefeldin A for 5 hours, and analyzed for their IL-17A and IFN-γ expression by flow cytometry. (**D**–**F**) Percentages of gated cells are shown.

IL-6–driven Th cells were encephalitogenic, regardless of their secretion of IL-17A (30). Also, the strains of mice used appear to determine the therapeutic potential of anti–IL-17A therapy. While IL-17 blockade has hardly any effect in C57BL/6 mice (23), SJL/J mice show more pronounced disease alleviation after anti–IL-17A treatment (9). Nevertheless, neutralization of IL-17A has not been demonstrated to completely prevent EAE development. Kroenke et al. recently reported that both Th1 and Th17 cells have encephalitogenic potential (31), while O'Connor and colleagues demonstrated that Th1 cells are required to initiate CNS inflammation and Th17 cells invade the CNS only later during disease (32).

By now, a whole family of new cytokines has been grouped around IL-17A, all sharing a distinct structural feature, a 4-cysteine knot (33). The closest associate to IL-17A is IL-17F (13, 14). Both cytokines are functionally related to neutrophil recruitment and expansion, angiogenesis, tissue remodeling, and the induction of proinflammatory factors, like IL-1β, TNF-α, chemokines, and defensins (11, 34, 35). We found that the loss of IL-17A coincided with consistently elevated levels of IL-17F expression, making IL-17F an ideal candidate to compensate for the loss of IL-17A in gene-targeted mice. We generated IL-17F–deficient mice and discovered that they are also fully susceptible to EAE and have no appreciable defect in generating an inflammatory response. One could argue conversely, that in *Il17f*$^{-/-}$ mice, IL-17A could compensate and that the actual function of *Il17a* and *Il17f* can only be appreciated in mice lacking both genes. Unfortunately, the close proximity of the loci of these 2 cytokines makes it virtually impossible to obtain double-deficient mice by mere interbreeding (approximately 44-kb distance between the 2 genes). To prevent any potential compensatory effect of IL-17A in *Il17f*$^{-/-}$ mice, we treated mice with antagonistic anti–IL-17A mAb as used previously by Langrish et al. (9). The fact that *Il17f*$^{-/-}$ mice treated with anti–IL-17A mAbs developed only a slightly ameliorated disease course, with a similar incidence and severity as untreated mice or WT mice, supports the notion that neither IL-17A nor IL-17F, either individually or in combination, are essential for the development of autoimmune CNS inflammation. In addition, the fact that mice in which transgenic overexpression of IL-17A is directed toward T cells also displayed an unaltered EAE phenotype further eliminates IL-17 as a key player in CNS autoimmunity. Integrating all current data generated by deletion of IL-17A as well as IL-17F and IL-22 (36) or induced overexpression of IL-17A by critical evaluation of its statistical versus biological relevance, we must conclude that, unlike IL-23, neither of these Th17 cytokines are key players in EAE. It is likely that the tissue distribution of

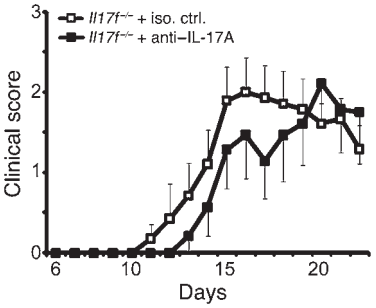

Figure 6
Loss of IL-17F and inhibition of IL-17A does not significantly impact on the development of EAE. EAE was induced in IL-17F–deficient mice by immunization with $MOG_{35–55}$/CFA. To antagonize IL-17A function, 200 μg of neutralizing anti–IL-17A mAb or the respective isotype control mAb was injected i.p. every fourth day, starting on day 4 after immunization. The graph shows the development of EAE according to clinical scores in 1 out of 2 independent experiments. Error bars represent mean ± SEM. Iso. ctrl., isotype control.

IL-17RA and IL-17RC, the receptor complex for IL-17A and IL-17F (17, 18), determines the pathogenic capacity of Th17 cells. The CNS expresses low levels of IL-17R in comparison to the skin or lung (37, 38), thus explaining the severe impact of total IL-17A overexpression on the skin but not the CNS. Translating these findings into the clinic for therapeutic targeting of this pathway in MS is even more complicated considering the data obtained in a recent clinical trial in MS patients (39). MS patients were treated with mAb specific to IL-12/IL-23p40, which surprisingly had no beneficial effect, indicating that in MS patients, these Th1- and Th17-inducing cytokines may not play an essential role in disease progression. On the other hand, targeting of the IL-23 pathway has a profound efficacious impact on psoriasis, again supporting the notion that the tissue distribution of cytokine receptors such as IL-17R determines the impact of Th17 cells on inflammation.

The mere presence of a proinflammatory molecule at the inflammatory site is often interpreted to convey a vital function ultimately to be translated into an attractive drug target. However, while we can clearly confirm that IL-17A and IL-17F have all the features of such a factor, in vivo targeting shows that this correlation is not in fact causative. While IL-17 family member molecules currently serve their purpose as markers for pathogenic self-reactive cells, we conclude that other, thus far, unidentified factors or mechanisms employed by Th17 cells must convey their pathogenic capacity. Alternatively, it is also possible that Th17 or Th1 cells harbor a small population of IL-23–driven T cells, which possess all the pathogenic potential, and that IL-17 gained its encephalitogenic role based upon the concept of "guilt by association".

Methods

Mice. Female C57BL/6 mice were purchased from Harlan Laboratories. *Il17a*$^{-/-}$ mice were generously provided by Yoichiro Iwakura (University of Tokyo, Tokyo, Japan). *Il17f*$^{-/-}$ (129 × C57BL/6) and IL-17A$^{ind/+}$ mice were generated as described in Supplemental Figure 1G. Animal experiments were approved by the Swiss Veterinary Office (Zurich, Switzerland) and the Central Animal Facility Institution of the University of Mainz.

Induction of EAE. For EAE induction, mice were immunized subcutaneously, with 200 μg of $MOG_{35–55}$-peptide emulsified in complete Freund's adjuvant supplemented with 2 mg/ml of *Mycobacterium tuberculosis*, into the lateral abdomen. On days 0 and 2 after immunization, 200 μg of pertussis toxin and PBS was administered intraperitoneally. Clinical disease was scored daily as follows: 0, no clinical disease; 1, limp tail; 2, impaired righting reflex; 3, hind limb paralysis; 4, moribund; 5, dead (4).

In vitro assays. Lymphocytes were cultured in RPMI1640 containing 10% FCS (both from Invitrogen). Restimulation of primed lymphocytes from LN, spleen, or cerebellum was measured at 7 days after immunization (pre-EAE) by $MOG_{35–55}$/CFA (4) and challenged with 50 μg/ml $MOG_{35–55}$ or 5 μg/ml concanavalin A. Proliferation was assessed by thymidine incorporation (40) and cytokine release by either FlowCytomix Th1/2 Multiplex (Bender MedSystems), ELISA (BD Biosciences — Pharmingen), or ELISPOT (2) as described. Activating anti-CD3 and anti-CD28 antibodies were used at concentrations of 1 μg/ml and 6 ng/ml, respectively. Fluorocytometric analysis of surface marker expression was performed as described (41). Intracellular cytokine staining was performed with the Cytofix/Cytoperm Plus Kit (BD Bioscience), according to manufacturers directions. The following antibodies were used: anti–IL-17A (TC11-18H10; BioLegend), anti–IFN-γ (XMG1.2; BD Biosciences — Pharmingen), and anti–IL-17F (R&D Systems). The IL-17F antibody was labeled using the Alexa Fluor 488 Monoclonal Antibody Labeling Kit (Invitrogen).

T cell polarization. For the in vitro generation of Th17 cells, splenocytes were harvested 7 days after immunization and were restimulated with 20 μg/ml $MOG_{35–55}$ (BioExpress), 5 ng/ml TGF-β, 20 ng/ml IL-6 (both from PeproTech EC), 10 ng/ml IL-23 (R&D Systems), 5 μg/ml anti–IFN-γ (R4-6A2; Bioexpress), and 5 ng/ml IL-2 (eBioscience). Anti–IFN-γ was added daily, and IL-23 was added on days 0 and 2. Cells were analyzed on day 6 after culture.

Real-time RT-PCR. RNA was extracted and cDNA prepared as described. The primers used for IL-17F were as follows: forward, CTGTTGATGTTGGGACTTGCC, and reverse, TCACAGTGTTATCCTCCAGG. β-actin and IL-17A primers were described elsewhere (2).

Histology and immunohistochemical staining. Spinal columns were fixed in 4% paraformaldehyde in PBS, paraffin embedded, cut, and stained with H&E, according to standard protocols. Immunohistochemical stainings on serial sections using antibodies to CNPase (1:500; Chemicon) and glial fibrillary acidic protein (1:4,000; DAKO) were carried out on an automated BenchMark Staining apparatus (Ventana Medical Systems), following the manufacturer's guidelines.

Extraction of mononucleated cells from inflamed CNS tissue and subsequent cytofluorometric analysis was performed as described previously (41).

Statistics. Clinical development was evaluated using a 2-tailed Student's *t* test as well as analysis of covariance analysis, using the R project for statistical computing. *P* values of less than 0.05 were considered significant.

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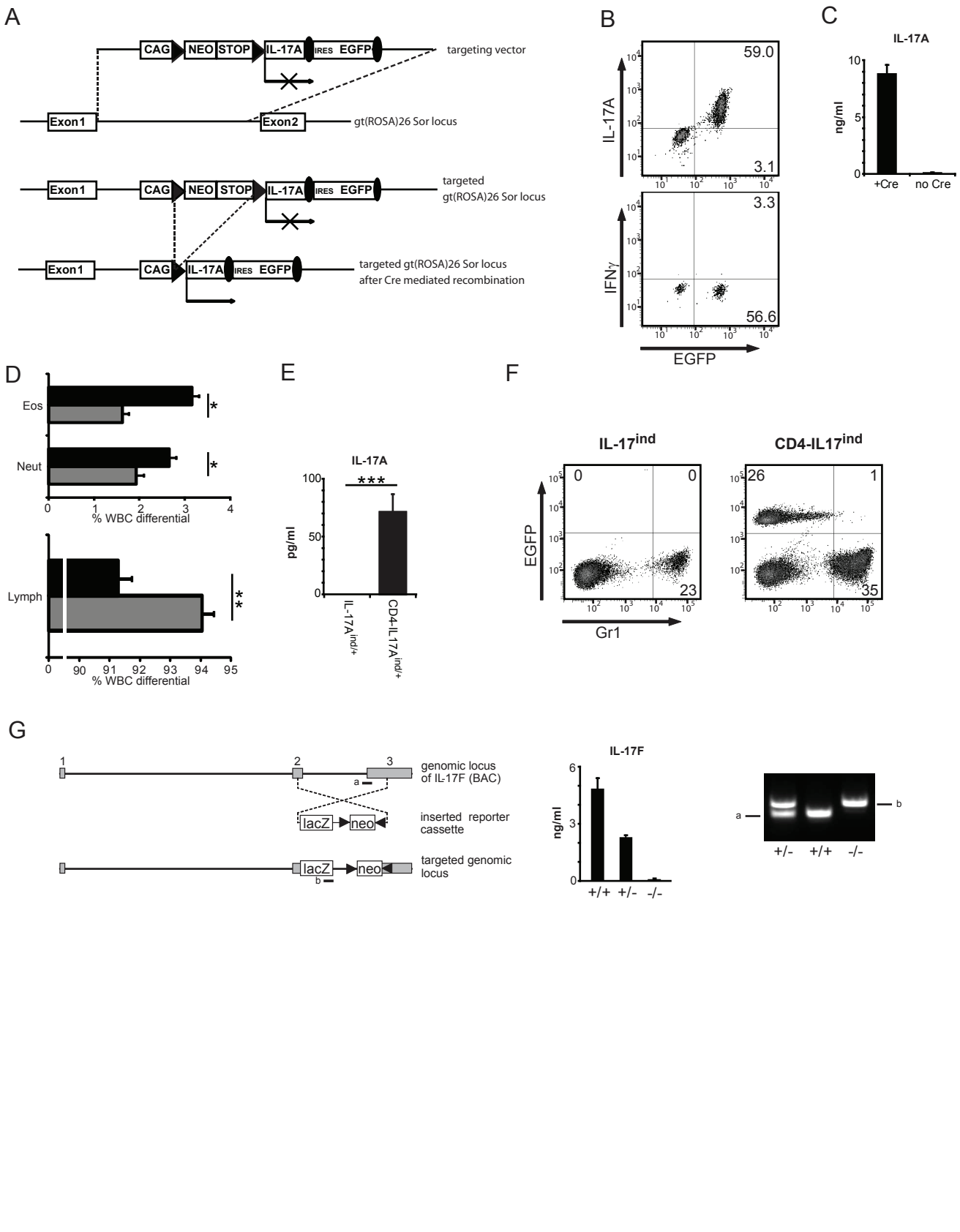
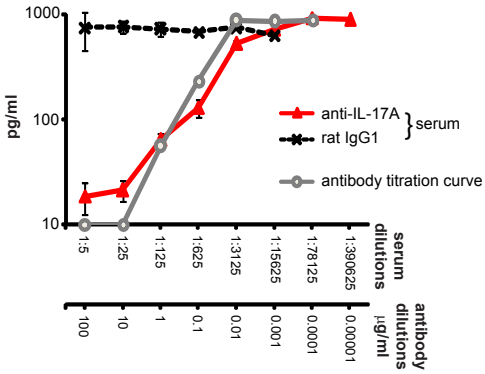
Supplemental Figure 1

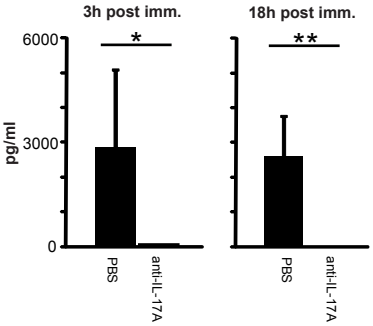


Supplemental Figure 2

A



B



Supplemental Figure 1 (A) Generation of the IL-17A^{ind} allele was carried out using homologous recombination in embryonic stem cells (C57BL/6-Bruce 4). Our conditional ‚knock-in' approach introduced the targeting construct into the endogenous gt(ROSA)26Sor locus. Upon Cre-mediated recombination, a lox-P-flanked transcriptional STOP cassette is excised 5' of an IL-17A cDNA insert and an IRES-EGFP element, allowing a dual expression of IL-17A and EGFP under the control of the chicken β-actin (CAG) promoter. (B) 1x10^6 whole lymph node cells from IL-17A$^{ind/+}$ mice were cultured for 4 h with or without (data not shown) Tat-Cre protein to induce IL-17A and EGFP expression in the presence of anti-CD3, anti-CD28 and IL-2. After 36 h, cells were briefly cultured with Brefeldin-A and stained for CD4 and either IL-17A or IFNγ. Percentages of IL-17A$^+$EGFP$^+$ and IFN-γ$^+$EGFP$^+$ cells are given in the quadrants. No EGFP was detected in wild type mice also treated with Tat-Cre (data not shown). (C) LN cells were cultured as in B, and supernatants extracted after 36 h. IL-17A secretion was measured by ELISA. (D) Peripheral blood was extracted from IL-17A$^{ind/+}$ and CD4-IL17A$^{ind/+}$ mice and subjected to routine WBC differential. Significant increase in neutrophil and eosinophil counts, and a decrease in peripheral blood lymphocytes are shown. (CD4-IL17A$^{ind/+}$ n=10, IL-17A$^{ind/+}$ n=9). (E) Serum from IL-17A$^{ind/+}$ and CD4-IL17A$^{ind/+}$ mice was extracted at d14 after immunization with MOG/CFA. Shown are levels of IL-17A detectable in serum isolated from peripheral blood (n=5). Spleen cells from the same mice were stained for Gr1. Quadrant statistics are shown. (F) Spleen-derived single cell suspension were obtained from the indicated genotypes d14 after MOG/CFA immunization. Percentages of Gr1$^+$ cells are shown in the histograms. (G) IL-17F gene-targeting strategy for the generation of the IL-17F deficient mouse strain. A reporter gene/resistance cassette (*lacZ* gene and a Ub1-Em7 promoter driven neomycin resistance gene, *neo*, flanked by loxP sites, ▶) was introduced into *il17f* exon 2 and 3 of a BAC containing the IL-17F gene locus. 129S6SvEv/C57B/6F1 embryonic stem cells were

targeted with a BAC carrying the replaced *il17f* gene locus and were subsequently screened as described elsewhere (41). “a” and “b” represent amplicons for the wild type and targeted allele respectively. Complete loss of IL-17F in the IL-17^{null} mouse was confirmed by ELISA on supernatant of T_H17 polarized splenocytes.

Supplemental Figure 2 Specification of the IL-17A blocking capacity of *in vivo* applied antagonistic antibody (A) WT or IL-17A$^{-/-}$ mice (n=3) were immunized with MOG/CFA and pertussis toxin. On day 0 and 4 either 200mg of antagonistic anti-IL-17A antibody or isotype control antibody (rat IgG1) was administered intraperitonially and mice are bled on day 7. The recovered serum was titrated in an IL-17A specific ELISA onto 1000 pg of IL-17A protein standard. The serum was serially diluted as shown in the graph. To estimate the anti-IL-17A titer in the serum the neutralizing antibody was itself in parallel titrated onto the same amount of IL-17A protein standard. IL-17A levels were measured after a 1 hour pre-incubation of the serum/antibody with the IL-17A standard. The graph shows the pooled data of all anti-IL-17A and rat IgG1 sera with SEM indicated. (B) Two groups of CD4-IL17A$^{ind/+}$ littermate mice were immunized with MOG/CFA (n=3). After 10 days, either 100μg/mouse of anti-IL-17A or sham antibody was injected intravenously. Mice were subsequently bled and serum was isolated either 3h or 18h after the antibody treatment. IL-17A serum concentrations were measured by flow cytometric cytokine analysis. Statistical significances are indicated in the graph (one-tailed T test).

Supplemental Table 1 Homeostatic leukocyte composition in spleen and thymus of naïve CD4-IL17A$^{ind/+}$ and control mice.

SPLEEN	Percentage	
	CD4-IL17A$^{ind/+}$	IL-17A$^{ind/+}$
T_H cells (CD4$^+$)	18	19
CTL (CD8$^+$)	9	11
T_{Reg} cells (FoxP3$^+$)	2	2
NK cells (NK1.1$^+$)	5	5
B cells (B220$^+$)	59	60
Macrophages (CD11b$^+$)	6	5
DC (CD11c$^+$)	6	4

THYMUS	Percentage	
	CD4-IL17A$^{ind/+}$	IL-17A$^{ind/+}$
T_H (CD4$^+$)	15	16
CTL (CD8$^+$)	6	7
Double positive T_H cells	73	73

Supplemental Table 2 Homeostatic leukocyte composition in spleen and thymus of naïve IL-17F deficient and control mice.

SPLEEN	Percentage		
	+/+	+/-	-/-
T_H cells (CD4$^+$)	14	16	14
CTL (CD8$^+$)	20	21	23
T_{Reg} cells (FoxP3$^+$)	3	3	2
NK cells (CD3$^-$, NK1.1$^+$)	3	4	3
NK T cells (CD3$^+$, NK1.1$^+$)	2	3	2
B cells	38	38	37
Macrophages (CD11b$^+$)	4	4	4
DC (CD11c$^+$)	4	4	3

THYMUS	Percentage		
	+/+	+/-	-/-
T_H (CD4$^+$)	14	15	13
CTL (CD8$^+$)	5	6	4
Double positive T_H cells	78	78	80

IL-22 Is Expressed by Th17 Cells in an IL-23-Dependent Fashion, but Not Required for the Development of Autoimmune Encephalomyelitis[1]

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Lately, IL-17-secreting Th cells have received an overwhelming amount of attention and are now widely held to be the major pathogenic population in autoimmune diseases. In particular, IL-22-secreting Th17 cells were shown to specifically mark the highly pathogenic population of self-reactive T cells in experimental autoimmune encephalomyelitis (EAE). As IL-17A itself was found to only play a minor role during the development of EAE, IL-22 is now postulated to contribute to the pathogenic function of Th17 cells. The goal of this study was to determine the role and function of IL-22 during the development of CNS autoimmunity in vivo. We found that CNS-invading encephalitogenic Th17 cells coexpress IL-22 and that IL-22 is specifically induced by IL-23 in autoimmune-pathogenic CD4$^+$ T cells in a time- and dose-dependent manner. We next generated IL-22$^{-/-}$ mice, which—in contrast to the prediction that expression of inflammatory cytokines by CNS-invading T cells inevitably confers pathogenic function—turned out to be fully susceptible to EAE. Taken together, we show that self-reactive Th cells coexpress IL-17 and IL-22, but that the latter also does not appear to be directly involved in autoimmune pathogenesis of the CNS. ***The Journal of Immunology,*** **2007, 179: 8098–8104.**

Multiple sclerosis (MS)[6] is the most common inflammatory disease of the CNS and its animal model experimental autoimmune encephalomyelitis (EAE) is mediated by the actions of autoreactive encephalitogenic Th cells. Although Th1 cells were long suspected to be the major pathogenic population, the discovery that IL-23, and not the Th1-inducing cytokines IL-12 and IL-18, is vital for EAE development initiated a major paradigm shift with regards to the role of Th1 cells in inflammation (1–5). The impact of IL-23 on Th cells appears to be restricted to memory cells, which in response to IL-23R engagement secrete IL-17 (5). IL-17 expression by T cells correlates superbly with an autoimmune-pathogenic phenotype and this polarization pattern was coined Th17 (6, 7). Although studying IL-23 under various inflammatory conditions lead to the discovery of Th17 cells, it was later found that the cytokines TGF-β and IL-6 are dominant in their capacity to polarize Th17 cells (8, 9). The role and function of IL-23 in maintaining this phenotype remains a subject of debate (5).

Th17 cells have received much attention lately and mice lacking IL-17A were found to be moderately resistant to EAE (10). However, in contrast to IL-17A$^{-/-}$ mice, IL-23-deficient mice are completely EAE resistant (1, 2). Thus, we reasoned that IL-17A is unlikely to be the only factor produced by Th17 cells involved in the inflammatory process. To identify the expression signature of IL-23-driven genes, we used high-density transcriptomics and identified IL-22 to be induced by IL-23 in autoimmune-pathogenic CD4$^+$ T cells in a time- and dose-dependent manner. IL-22 belongs to the IL-10 superfamily of cytokines and exhibits—unlike IL-10—potent proinflammatory properties. Its recently reported role in psoriasis (11–13) combined with our finding that IL-22 is specifically induced by IL-23 points toward a relevant function of IL-22 in autoimmune inflammatory diseases. Bettelli et al. (14) further reported that IL-22 marks a particularly pathogenic population of autoreactive T cells implicating IL-22 as a major pathogenic cytokine during CNS inflammation. In addition the *IL-22* gene, together with IL-26 and IFN-γ on the human chromosome 12q14, are considered a prominent susceptibility locus for MS (15). We found that following IL-23 stimulation, IL-22 is specifically secreted by pathogenic Th cells. To determine the actual role

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[6] Abbreviations used in this paper: MS, multiple sclerosis; EAE, experimental autoimmune encephalomyelitis; MOG, myelin oligodendrocyte glycoprotein; Tg, transgenic; wt, wild type; LN, lymph node; BM, bone marrow; DC, dendritic cell.

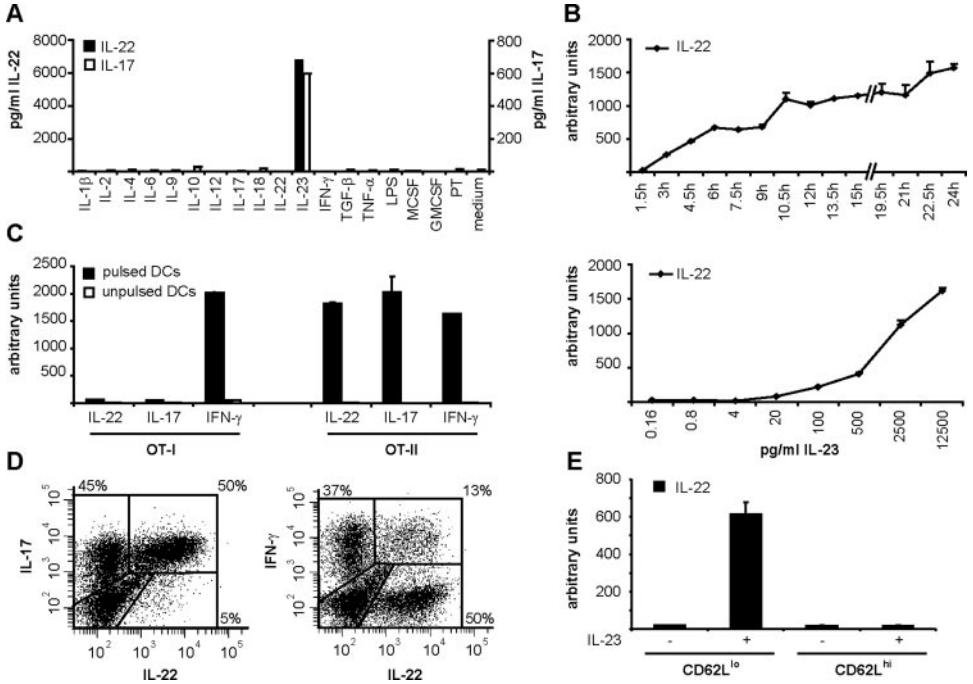

FIGURE 1. IL-22 expression is specifically induced by IL-23. *A*, A total of 4×10^6 splenocytes were obtained from C57BL/6 mice, stimulated with 20 ng/ml of the indicated substances, and incubated for 24 h. The supernatant was collected and used for IL-22 and IL-17A protein detection by ELISA. *B*, A total of 4×10^6 naive wt splenocytes were stimulated with 20 ng/ml IL-23 and collected at indicated time points or stimulated with indicated concentrations of IL-23 and harvested after 24 h. RNA was extracted, and quantitative RT-PCR for IL-22 mRNA expression performed. *C*, A total of 4×10^6 $CD8^+$ or $CD4^+$ T cells were purified from OT-I or OT-II splenocytes, respectively, and incubated with cognate peptide pulsed or unpulsed BM-derived DCs for 48 h and harvested after 24 h. RNA was extracted, and quantitative RT-PCR for IL-22, IL-17, and IFN-γ mRNA expression performed. All data shown are representative of at least three individual experiments and SD were calculated from duplicate wells. *D*, Splenocytes from a MOG-immunized C57BL/6 mouse were activated in vitro 7 days postinfection with 15 μg/ml MOG peptide, 10 ng/ml IL-23, 20 ng/ml IL-6, 5 ng/ml human TGF-β, IL-7, and IL-2 and 5 μg/ml anti IFN-γ Ab to generate Th17 cells. Intracellular cytokine staining was performed on day 5. The plot is gated on $CD45^+CD4^+$ cells. Shown are the percentages only of polarized T cells. *E*, Splenocytes were obtained from C57BL/6 mice and MACS sorted for $CD4^+CD62L^{high}$ and $CD4^+CD62L^{low}$. A total of 4×10^6 cells were stimulated with 20 ng/ml IL-23 and harvested after 24 h. RNA was extracted, and quantitative RT-PCR for IL-22 mRNA expression performed. Data shown are representative of three experiments.

of this cytokine in autoimmune inflammation, we generated IL-$22^{-/-}$ mice, which were found to be surprisingly fully susceptible to EAE. We show that self-reactive Th cells coexpress IL-17 and IL-22, but that the latter does not appear to be directly involved in autoimmune pathogenesis of the CNS.

Materials and Methods

Peptides, Abs, and recombinant cytokines

Myelin oligodendrocyte glycoprotein $(MOG)_{35–55}$ peptide (MEVGWYR SPFSRVVHLYRNGK) and $OVA_{323–339}$ (ISQAVHAAHAEINEAGR) were obtained from Research Genetics. All recombinant cytokines were purchased from PeproTech and all Abs were purchased from BD Biosciences. The Ab to murine IL-22 was provided by Genentech and labeled with Alexa 488 (Invitrogen Life Technologies) according to the manufacturer's directions.

Mice and induction of EAE

C57BL/6 mice, IL-12 $p35^{-/-}$, and IL-12 $p40^{-/-}$ mice on a C57BL/6 background were purchased from The Jackson Laboratory and were bred under specific pathogen-free conditions. The 2D2 (MOG-TCR-transgenic (Tg)) mice were provided by V. Kuchroo (Harvard Medical School, Boston, MA). IL-$22^{-/-}$ mice were generated by targeting exons 1–3 and backcrossed onto C57BL/6 for more than eight times. The targeting vector was constructed to replace the exons 1a, 1b, 2, and a part of exon 3 of the *IL-22a* gene by a neomycin-resistant gene. A 5′ arm of 1521 bp was amplified using a mutated sense primer with a *Xho*I site 5′-CTTCGGCTCG AGATGGCCAC-3′ a mutated antisense primer containing also a *Xho*I site 5′-GCCCTCGAGACACCAGGGTT-3′ to allow the direct insertion into the pPNT vector. The 3′ arm consisted of a 3559-bp *Kpn*I fragment, containing the end of exon 3 and exon 4, and was cloned. For genotyping, the targeted gene was amplified using a sense primer located upstream the 5′ arm: 5′-CTGCTGTCCAACAGAGCTCT-3′ and antisense primer on neomycin gene: 5′-CGCCTCCCCTACCCCGGTAGA-3′, resulting in a 1.7-kb amplified sequence. The wild-type (wt) gene was amplified using a sense primer located into the 5′ arm 5′-AATCTATGAAGTTGGTGG GA-3′ and an antisense primer located on exon 2 5′-ACTGACTCCTCG GAACAGTT-3′, resulting in a 1.2-kb amplified sequence. Mouse IL-22 RT-PCR was performed as previously described (16). EAE was induced and scored as described (17).

Histology and flow cytometry

Whole mouse brains or spinal columns were fixed in 4% paraformaldehyde in PBS, paraffin-embedded, cut and stained with H&E and Luxol-Nissl according to standard protocols. Immunohistochemical stainings on serial sections using Abs to neurofilament protein (NF, 200 kDa subunit; 1:20; Bio-Science), Iba1 (1:100; Wako Chemicals), CD3 (1:150; Labvision), and B220 (1:1000; BD Biosciences) were conducted on an automated Nexus staining apparatus (Ventana Medical Systems), following the manufacturer's guidelines.

CNS-infiltrating lymphocytes were isolated as described previously (4). For flow cytometry, Abs were incubated with cells for 20 min at 4°C and then cells were analyzed with a FACSCalibur (BD Pharmingen) and FACSDiva software. Postacquisition analysis was done with FACSDiva (BD Pharmingen) or FlowJo7 software (Tree Star). For intracellular cytokine staining, cells were restimulated with 50 ng/ml PMA, 500 ng/ml ionomycin, and GolgiPlug (BD Biosciences) for 5 h. Cells were first stained for surface Ags and then permeabilized with Cytofix/Cytoperm (BD Biosciences) according to the manufacturer's recommendations. Intracellular cytokine staining was performed using Abs to IFN-γ, IL-17A, or IL-22 as described above.

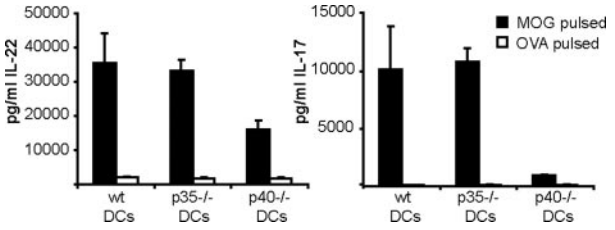

FIGURE 2. IL-22 and IL-17 are produced by MOG-reactive T cells upon encounter with their cognate Ag. T cells from mice Tg for the *2D2* TCR were isolated and stimulated with MOG_{35-55} or control peptide (5 μg/ml) pulsed mature BM-derived DCs obtained from wt, $p35^{-/-}$, or $p40^{-/-}$ mice for 2 days and IL-22 and IL-17A protein expression was measured by ELISA. Shown is a representative of three individual experiments ($n = 4$).

Cell culture and in vitro assays

Mice were sacrificed using CO_2, axillary and inguinal lymph nodes (LN) and spleens were collected and treated with 0.5 mg/ml DNase and 1 mg/ml Liberase (Roche) for 30 min at 37°C. Cells were cultured in RPMI 1640 supplemented with 10% FCS, 50 U/ml penicillin, and 50 μg/ml streptomycin (Invitrogen Life Technologies) in the presence or absence of the factors indicated in the figure legends and harvested at indicated time points. Where indicated, T cells were purified from splenocytes by magnetic cell sorting with MACS Beads following the manufacturer's recommendation (Miltenyi Biotec).

Bone marrow (BM)-derived dendritic cells (DCs) were generated as described (4). To mature DCs, 10 μg/ml LPS (Fluka) was added to the culture for 24 h. Mature DCs were pulsed with 5 μg/ml peptide for 4 h, washed extensively, and incubated with splenocytes at a ratio of 1:4 and harvested after 48 h.

Cytokine analysis

ELISA for IL-17A (BD Pharmingen) and IL-22 (Antigenix) were performed according to the manufacturer's instructions. Proliferation of MOG-reactive cells were stimulated in triplicate for 48 h with either 50 μg/ml MOG_{35-55}, 5 μg/ml Con A, or medium, and 0.5 μCi/ml [^{3}H]thymidine was added after 24 h for assessment of proliferative responses. Thymidine incorporation was assessed with a Filtermate Collecter (Applied Biosystems) and a scintillation and luminescence counter.

Real-time RT-PCR

Cells or tissues were homogenized in 1 ml of TRIzol reagent (Invitrogen Life Technologies). Total RNA was extracted and reverse transcription was performed using random hexamer primer and Moloney murine leukemia virus reverse transcriptase (Invitrogen Life Technologies). After PCR amplification using SYBR Green PCR master mix (Invitrogen Life Technologies), quantitative values of each sample were normalized to its β-actin content and converted to relative cDNA quantities by comparison to a standard curve generated with dilutions of β-actin plasmid. Primers were purchased from Operon Technologies. The primers used were: (5′–3′) β-actin forward (fw): agagggaaatcgtgcgtgac, β-actin reverse (rev): caatag tgatgacctggccgt; IL-22 fw: ttgaggtgtccaacttccagca, IL-22 rev: agccggacgtc tgtgttgtta; IL-17 fw: atcaggacgcgcaaacatga, IL-17 rev: ttggacacgctgag ctttga.

Results

IL-23 induces IL-22 *gene expression*

To elucidate the identity of IL-23-driven gene transcripts, we devised two reciprocal approaches for whole genome transcriptomics. We compared gene expression induced by IL-23 stimulation with those absent in Ag-driven IL-23-deficient ($p40^{-/-}$) lymphocytes. In the first approach, genes up-regulated (>4-fold) by IL-23 were identified by stimulating splenocytes obtained from an unmanipulated mouse with recombinant IL-23 or IL-12 as a control. In the second approach, we immunized wt, $IL\text{-}12p35^{-/-}$, and $IL\text{-}12/23p40^{-/-}$ mice with keyhole limpet hemocyanin and 7 days postinfection harvested lymphocytes and rechallenged them in vitro with keyhole limpet hemocyanin before harvesting the mRNA for microchip analysis (Affymetrix chip MOE430A). We used IL-12 as a control, first because IL-12-induced gene expression is well-characterized, and second to eliminate IL-12-induced

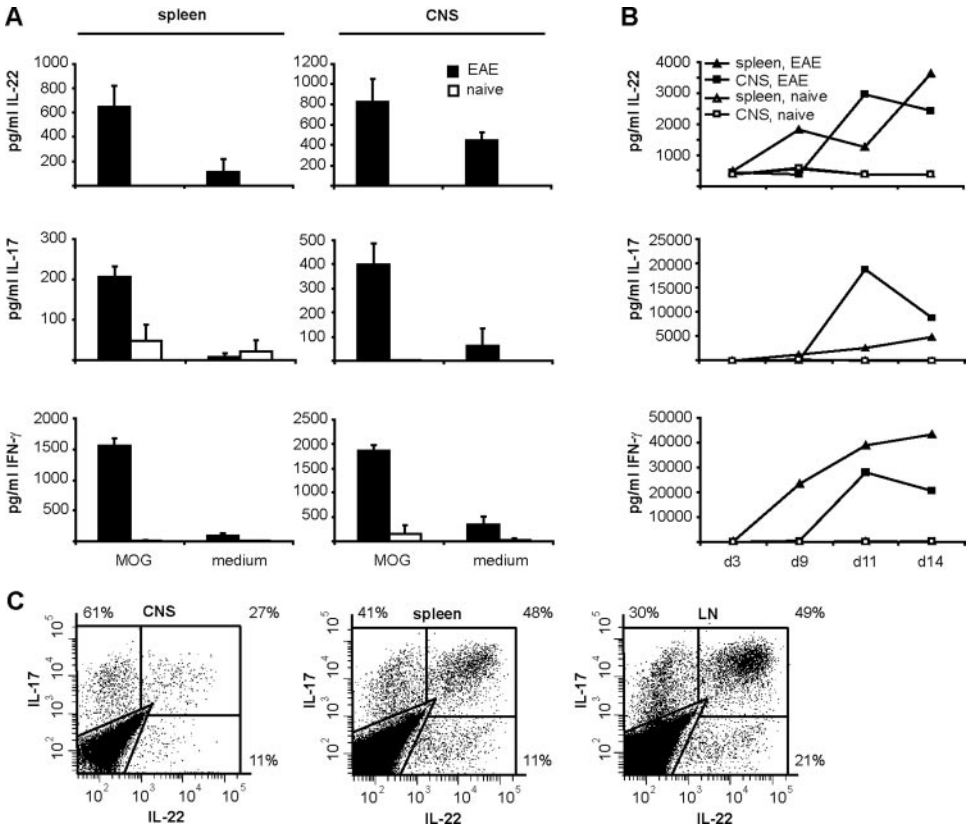

FIGURE 3. CNS-infiltrating Th17 cells express IL-22. Wild-type mice were immunized with MOG_{35-55} emulsified in CFA and sacrificed either (*A* and *C*) at the peak of disease (day 21) or (*B*) at indicated time points after immunization. Lymphocytes were isolated out of CNS and spleen or LN of naive or MOG_{35-55}-immunized wt mice, restimulated with 50 μg/ml of their cognate Ag MOG_{35-55}, and IL-22, IL-17A, and IFN-γ expression was analyzed by (*A*) ELISA or (*C*) intracellular cytokine staining. *A*, A representative ELISA of four individual experiments ($n = 2$/group/experiment). *B*, Pre-onset disease was performed with $n = 3$. *C*, The percentages only of polarized T cells. Shown is a representative of three individual experiments, $n = 3$.

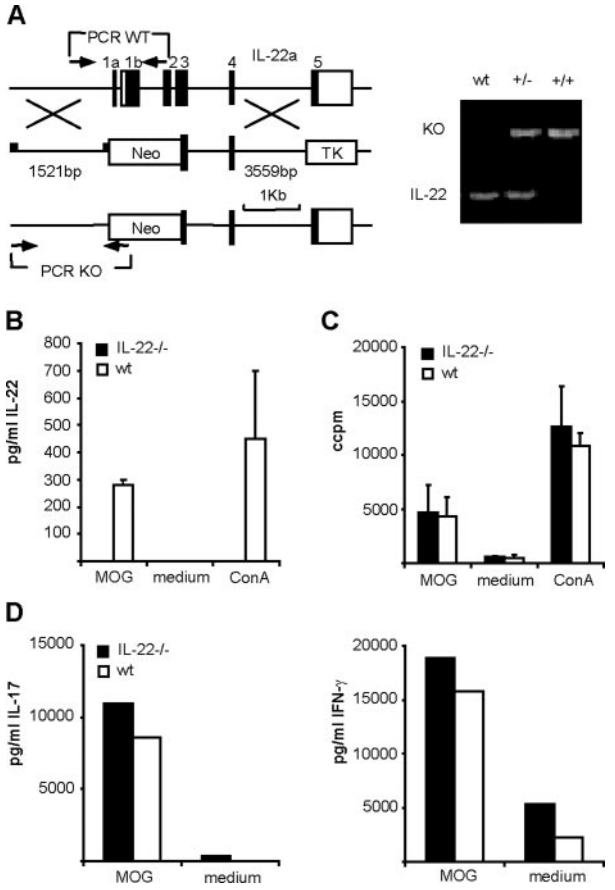

FIGURE 4. Generation and characterization of IL-22$^{-/-}$ mice. *A*, The structure of the *IL-22a* locus, the targeting vector, and the predicted homologous recombination are shown. The exons are shown as boxes, with white and black boxes for noncoding and coding regions, respectively. The size of the 5′ and 3′ arms, as well as the location of the primers used for genotyping are indicated. Neo, neomycin-resistance cassette; TK, thymidine kinase cassette. For genotyping, the wt and targeted alleles were amplified from F_2 tail genomic DNA as described in *Materials and Methods.* Lymphocytes were isolated out of spleen of MOG_{35-55} immunized wt or IL-22$^{-/-}$ mice, restimulated with 50 μg/ml of their cognate Ag MOG_{35-55}, or 5 μg/ml ConA. *B*, IL-22 expression was analyzed by ELISA. *C*, Proliferation was measured by thymidine uptake (shown is a representative of three individual experiments $n = 3$). *D*, IL-17A and IFN-γ expression was analyzed by ELISA. Data shown are representative of at least three individual experiments and SD were calculated from duplicate wells.

target genes from our analysis. By combining both data sets, we found IL-22 to be specifically and strongly induced by IL-23 (data not shown).

To verify that IL-22 expression is specifically induced by IL-23, we treated splenocytes derived from unmanipulated C57BL/6 mice with an array of different stimuli, harvested the mRNA and measured IL-22 and IL-17A protein expression by ELISA (Fig. 1*A*). Other than IL-23, none of the used substances elicited significant levels of IL-22 and IL-17 expression in splenocytes after 24 h of stimulation. Different concentrations of the different stimuli were used (data not shown). Our data show that a population of splenocytes present in naive pathogen-free C57BL/6 mice respond to IL-23R engagement with IL-22 and IL-17 expression. To further characterize the kinetics and dose dependence of IL-23-induced IL-22 production, we stimulated wt lymphocytes obtained from an untreated mouse with IL-23 for different periods of time or in the presence of different concentrations of IL-23 and observed that IL-22 expression is induced in a time- and dose-dependent manner

Table I. *Distribution of cell types of the immune system in C57BL/6 and IL-22$^{-/-}$ mice: spleen*[a]

Spleen	Percentage	
	wt	IL-22$^{-/-}$
Th cells (CD4$^+$)	18	16
CTLs (CD8$^+$)	11	10
Double-positive T cells (CD4$^+$CD8$^+$)	0.5	0.5
Regulatory T cells (FoxP3$^+$)	0.5	0.4
NK cells (CD3$^-$NK1.1$^+$)	2	1.9
NKT cells (CD3$^+$NK1.1$^+$)	2.1	1.9
B cells (B220$^+$)	62	65
Macrophages (CD11b$^+$)	4	3
DCs (CD11c$^+$)	3	3
Naive T cells (CD3$^+$CD62L$^+$)	25	23
Memory T cells (CD3$^+$CD62L$^-$)	5	6

[a] Spleens were obtained from unmanipulated C57BL/6 and IL-22$^{-/-}$ mice; cells were isolated and stained with fluorochrome-conjugated Abs against the indicated cell-type-specific surface markers. Data shown are representative of three individual experiments.

(Fig. 1*B*). We observed a similar expression pattern with IL-17 (data not shown). To verify the notion that Th cells and not CTLs are the main source of IL-22, we stimulated purified CD4$^+$ as well as CD8$^+$ T cells obtained from OVA TCR-Tg mice (OT-II and OT-I, respectively) with cognate peptide pulsed DCs for 24 h and found that only TCR-Tg CD4$^+$ T cells made IL-22 (Fig. 1*C*). By intracellular cytokine staining of Th17 cells, we could show that >90% of IL-22-secreting cells also produce IL-17, while fewer IFN-γ-secreting cells coexpress IL-22 (20%) (Fig. 1*D*). To identify whether IL-23 stimulates the secretion of IL-22 by naive or memory T cells, we purified memory T cells (CD62L^{low}) and naive T cells (CD62L^{high}) followed by an overnight stimulation with IL-23. Our data confirm that IL-23 primarily drives the memory T cell pool and does not influence the naive pool in regards to cytokine secretion measured (Fig. 1*E*).

IL-22 is expressed by encephalitogenic Th cells

To determine the induction of IL-22 and IL-17A expression in a more physiologic manner in response to cognate Ag, we isolated T cells from MOG-reactive TCR-Tg (2D2) mice and stimulated them with MOG_{35-55} or control peptide-pulsed mature BM-derived DCs obtained from wt, IL-12p35$^{-/-}$, or IL-12/23p40$^{-/-}$ mice. IL-22 and IL-17A expression was subsequently measured by

Table II. *Distribution of cell types of the immune system in C57BL/6 and IL-22$^{-/-}$ mice: lymph node*[a]

LN	Percentage	
	wt	IL-22$^{-/-}$
Th cells (CD4$^+$)	37	37
CTLs (CD8$^+$)	27	27
Double-positive T cells (CD4$^+$CD8$^+$)	1.2	1.7
Regulatory T cells (FoxP3$^+$)	0.3	0.2
NK cells (CD3$^-$NK1.1$^+$)	0.5	0.8
NKT cells (CD3$^+$NK1.1$^+$)	0.4	0.6
B cells (B220$^+$)	33	35
Macrophages (CD11b$^+$)	3	3
DCs (CD11c$^+$)	1	1
Naive T cells (CD3$^+$CD62L$^+$)	64	62
Memory T cells (CD3$^+$CD62L$^-$)	9	9

[a] LNs were obtained from unmanipulated C57BL/6 and IL-22$^{-/-}$ mice; cells were isolated and stained with fluorochrome-conjugated Abs against the indicated cell-type-specific surface markers. Data shown are representative of three individual experiments.

Table III. *Distribution of cell types of the immune system in C57BL/6 and IL-22$^{-/-}$ mice: thymus*[a]

Thymus	Percentage	
	wt	IL-22$^{-/-}$
Th cells (CD4$^+$)	7	7
CTLs (CD8$^+$)	3	3
Double-positive T cells (CD4$^+$CD8$^+$)	87	83

[a] Thymi were obtained from unmanipulated C57BL/6 and IL-22$^{-/-}$ mice; cells were isolated and stained with fluorochrome-conjugated Abs against the indicated cell-type-specific surface markers. Data shown are representative of three individual experiments.

ELISA. MOG-reactive T cells clearly expressed high levels of IL-22 and IL-17A after encounter with their cognate Ag (Fig. 2). This response is dependent on IL-23 as reduced levels of IL-22 and IL-17 were detectable when T cells were cocultured with DCs obtained from IL-12/23p40$^{-/-}$ mice. We have also performed this restimulation experiment using polyclonal effector T cells isolated from MOG-immunized wt mice and confirmed that the expression of IL-22 and IL-17 is dependent on the secretion of IL-23 by APCs (data not shown).

To analyze the expression of IL-22 in mice with autoimmune inflammation, we induced EAE in wt C57BL/6 mice through immunization with $MOG_{35–55}$ emulsified in CFA and harvested brain and spinal cord as well as splenocytes at the peak of disease (day 21). Lymphocytes were isolated as described, challenged with $MOG_{35–55}$, and analyzed for cytokine expression after 48 h by ELISA (Fig. 3*A*). As expected, we found IFN-γ and IL-17 to be expressed by splenocytes and CNS-invading lymphocytes. Most importantly, we detected a significant production of IL-22 by encephalitogenic, CNS-infiltrating lymphocytes after re-encounter with their cognate MOG Ag in vitro. Kinetic analysis of IL-22 secretion was performed by sacrificing mice at different time

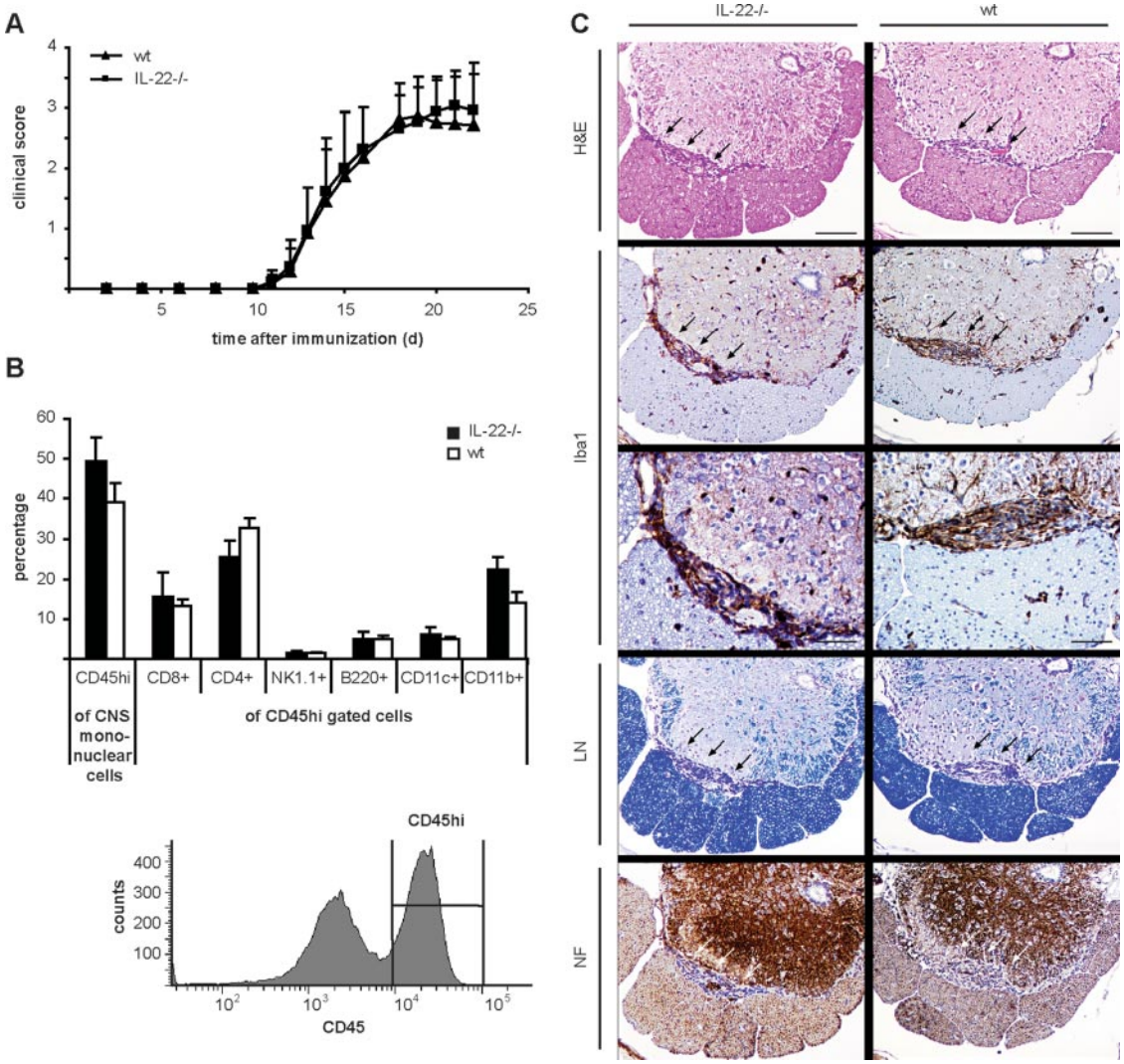

FIGURE 5. IL-22$^{-/-}$ mice are fully susceptible to EAE. *A*, IL-22$^{+/+}$ and IL-22$^{-/-}$ mice were immunized with $MOG_{35–55}$ emulsified in CFA and the clinical score recorded as described. Two merged representative experiment ($n \geq 12$) of five individual experiments are shown. Error bars represent the average deviation. Statistical significance was determined using an unpaired Student *t* test. *B*, Mice were sacrificed at the peak of disease (day 22), lymphocytes were isolated out of CNS, stained for the indicated surface markers and analyzed by flow cytometry. The histogram represents a staining for CD45 to distinguish CNS-resident microglia (CD45^{low}) from CNS-invading leukocytes (CD45^{high}). Shown is an average of five individually analyzed mice ± average deviation. *C*, Spinal cord cross-sections of wt and IL-22$^{-/-}$ mice displayed similar inflammatory lesions (arrows), which often appeared to be accentuated around vessels. They consisted of many activated Iba1$^+$ macrophages/microglia (second and, at higher magnification, third row) and caused an impairment of myelinated and axonal structures, as depicted by a Luxol-Nissl (LN; fourth row) as well as a neurofilament (NF) stain (fifth row). Scale bars: 100 μm for first, third, fourth, and fifth column; 100 μm for second, fourth, and sixth column.

Table IV. *IL-22$^{-/-}$ are fully susceptible to EAE*[a]

Genotype	wt	IL-22$^{-/-}$
Incidence	21 of 22	19 of 19
Day of disease onset	13.3 ± 1.78	13.7 ± 2.22
Maximum clinical score	2.93 ± 0.44	3.17 ± 0.46

[a] IL-22$^{+/+}$ and IL-22$^{-/-}$ mice were immunized with MOG_{35-55} emulsified in CFA and the clinical score was recorded as described. Three merged representative experiments ($n \geq 19$) of five individual experiments are shown. Onset and maximum clinical score were calculated ± average deviation. Statistical significance was determined using an unpaired Student's *t* test.

points after immunization with MOG/CFA. Similar to IFN-γ and IL-17, IL-22 expression by CNS-infiltrating lymphocytes increased with disease severity (Fig. 3*B*). To study which population of polarized Th cells secrete IL-22 in peripheral organs and the inflamed CNS, we immunized C57BL/6 mice with MOG/CFA and harvested spleen, LNs, and CNS at the peak of clinical EAE (average score of 3). The mononuclear cells were then restimulated with MOG_{35-55} followed by intracellular cytokine staining. Cytofluorometric analysis revealed that in spleen and LN, there is a high overlap of IL-17- and IL-22-secreting T cells, while in the inflamed CNS, IL-17-secreting cells dominate over IL-22 and IL-17/22-secreting T cells (Fig. 3*C*).

Gene targeting of IL-22 does not prevent EAE development

Given the clear expression pattern of IL-22 associated with pathogenic Th17 cells, we sought to investigate whether IL-22 plays a role in inflammation of the CNS. To do so, we generated IL-22$^{-/-}$ mice by replacing the coding exons 1a, 1b, 2, and a part of exon 3 of the *IL-22* gene with a neomycin-resistant gene (Fig. 4*A*) and verified the absence of IL-22 by genomic PCR, RT-PCR, and ELISA (Fig. 4, *A* and *B*, and data not shown). The mice do not display any obvious malformation of the hemopoietic system and developed normally (Tables I–III). When we analyzed the proliferating capacity as well as the cytokine expression profile of IL-22$^{-/-}$ cells after re-encounter with MOG_{35-55} by thymidine incorporation or ELISA, respectively, we observed that they behaved similar to wt cells (Fig. 4, *C* and *D*).

We induced EAE in the IL-22$^{-/-}$ mice through immunization with MOG/CFA and recorded the clinical disease development. Despite our expectations that IL-22 would display proinflammatory encephalitogenic properties, we observed that IL-22$^{-/-}$ mice developed disease similar to wt controls (Fig. 5*A* and Table IV). Cytofluorimetric analysis of CNS invading mononucleated cells revealed that their numbers and subset distributions are indistinguishable between the inflamed CNS of wt and IL-22KO mice (Fig. 5*B*). Histological analysis of spinal cord cross-sections further displayed similar inflammatory lesions consisting of activated Iba1$^+$ macrophages/microglia, CD3$^+$ T cells, and few B220$^+$ B cells resulting in an impairment of myelinated and axonal structures (Fig. 5*C* and data not shown). Therefore, while IL-22 is clearly expressed by encephalitogenic Th17 cells, it does not appear to be crucial for the development of autoimmune inflammation of the CNS.

Discussion

In the recent past, the function, origin, and regulation of IL-17-expressing Th cells received much attention by the immunology community. The discovery of this Th-polarization profile (Th17) finally does resolve a number of conflicting findings regarding the Th1/2 paradigm of tissue-directed autoimmune disease (5). The expression of IL-17 by Th cells correlates extremely well with their pathogenicity during autoimmunity.

However, despite this close correlation (3, 6, 7), several questions regarding their actual effector function remain unanswered. Foremost, the fact that IL-23 is absolutely vital for the development of autoimmune disease, whereas IL-17A alone has only a moderate impact (10), raises the question whether additional thus far unidentified IL-23-driven cytokines have pathogenic properties. We sought to resolve this question by the global analysis of IL-23-induced genes in lymphocytes. We discovered IL-22 to be the most prominent gene expressed by Th cells after IL-23 treatment. We further found that self-reactive Th cells required the presence of IL-23 for IL-22 production and that IL-23-deficient APCs were not able to properly induce IL-22 by stimulation of a population of MOG-reactive T cells. In agreement with Liang et al. (18), we found IL-22 to be highly expressed by Th17 cells. This suggested that IL-22 could potentially serve a pathogenic function during EAE. To this end, we performed a longitudinal analysis of IL-22 expression during EAE and found a strong correlation between T cell pathogenicity and IL-22 secretion. Bettelli et al. (14) recently claimed that IL-22 expression "marks" a highly pathogenic and proinflammatory population of autoaggressive T cells, heavily implicating IL-22 to exert a pathogenic function during EAE. Also, the receptor for IL-22, a heterodimer of the IL-10R2 and IL-22R1, like the IL-17A receptor is found primarily on stroma cells including endothelial cells, epithelial cells, and CNS-resident astrocytes (12, 14, 19). The close association of IL-22 and IL-17 in pathogenic Th cells, their inducibility by IL-23, and the fact that their receptors are expressed by similar cell types, implies that IL-22 too serves a proinflammatory pathogenic role in CNS inflammatory disease.

To determine whether IL-22 actually contributes to the development of EAE or whether the crisp correlation between IL-22 expression and encephalitogenicity is only an epiphenomenon, we generated IL-22$^{-/-}$ mice by gene targeting. To our surprise, we discovered that IL-22$^{-/-}$ mice develop EAE with the same severity, day of onset, and clinical manifestations as wt mice. This finding clearly dismisses IL-22 as a major pathogenic player in the development of autoimmune CNS inflammation. The function of IL-22 in autoimmunity, however, cannot be dismissed altogether. Wolk et al. (11) reported that elevated levels of IL-22 can be found in the blood of psoriatic patients and ear-skin acanthosis and inflammation induced by the application of IL-23 is slightly decreased when IL-22 is absent (11, 13).

Taken together, the notion that a cytokine is considered to have pathogenic functions cannot be based on its mere presence in a potentially pathogenic population of T cells. This line of thought had lead to a biased interpretation of the role and function of Th1 and Th2 cells in the context of autoimmune disease (5, 20–22). We were able to identify such a proinflammatory factor, namely IL-22, which is like IL-17A closely associated with an encephalitogenic phenotype. However, the fact that IL-22$^{-/-}$ mice develop severe EAE indicates that IL-22, just like IFN-γ, is not among the proinflammatory factors mediating the tissue damage seen in EAE. The requirement of the transcription factors which drive Th1 and Th17 polarization (T-BET and ROR-γT, respectively) indicates that features, other than the main cytokines produced, are responsible for their pathogenic behavior. Although Th17 cells secrete IL-17 as well as IL-22, the report by Kebir et al. (23) suggests that cytolytic enzymes and factors that alter the integrity of the blood-brain barrier may be responsible for the encephalitogenicity of human Th17/22 cells. It is however likely that among the genes expressed by Th17 cells, a number of them may turn out to serve as biomarkers if not

therapeutic targets in the treatment of autoimmune diseases in general and MS in particular.

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Disclosures

The authors have no financial conflict of interest.

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SHORT COMMUNICATION

IL-23-driven encephalo-tropism and Th17 polarization during CNS-inflammation *in vivo*

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IL-23 but not IL-12 is essential for the development of autoimmune tissue inflammation in mice. Conversely, IL-12 and IL-23 impact on the polarization of Th1 and Th17 cells, respectively. While both polarized T helper populations can mediate autoimmune inflammation, their redundancy in the pathogenesis of EAE indicates that IL-23 exerts its crucial influence on the disease independent of its T helper polarizing capacity. To study the impact of IL-23 and IL-12 on the behavior of encephalitogenic T cells *in vivo*, we generated BM-chimeric mice in which we can trace individual populations of IL-23 or IL-12 responsive T helper cells during EAE. We observed that T cells, which lack IL-12Rβ1 (no IL-12 and IL-23 signaling), fail to invade the CNS and do not acquire a Th17 phenotype. In contrast, loss of IL-12 signaling prevents Th1 polarization but does not prevent T-cell entry into the CNS. The loss of IL-12R engagement does not appear to alter T-cell expansion but leads to their accumulation in secondary lymphoid organs. We found that IL-23 licenses T cells to invade the target tissue and to exert their effector function, whereas IL-12 is critical for Th1 differentiation, but does not influence the pathogenic capacity of auto-reactive T helper cells *in vivo*.

Key words: Autoimmunity · EAE · IL-17 · IL-23 · T helper cells

See accompanying commentary by Segal

Supporting Information available online

Introduction

EAE is an inflammatory disease of the CNS that is induced in susceptible animals by immunization with myelin proteins or peptides. It serves as a reliable animal model for CNS-inflammation and autoimmunity [1]. The disease is the result of a $CD4^+$ T-cell-mediated immune response directed at the myelin sheath within the CNS. For many years Th1 cells were believed to be the most encephalitogenic population of T cells [2]; more recently Th17 cells were claimed to have more pathogenic potential than Th1 cells [3]. While the inflammatory signature and regulation of Th17 cells have been studied in depth, a single key molecule produced by Th17 cells and responsible for the encephalitogenic properties of T helper cells remains to be discovered [4, 5]. Numerous studies had inadvertently indicated the pivotal role of the cytokine IL-12 in the pathogenesis of EAE, as eliminating either the IL-12Rβ1 subunit of the receptor or the p40 subunit of the cytokine renders mice resistant to EAE [6]. However, unexpectedly the deletion of the second IL-12 subunit, p35 or the signaling subunit of the receptor, IL-12Rβ2, has either no impact on disease susceptibility or results in an even more severe clinical outcome [7, 8]. The discovery of IL-23 provided an explanation for the discrepancies observed in studies performed with p40- and p35-deficient animals as IL-12 and IL-23 share subunits and receptor

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components. IL-23 is a heterodimeric protein composed of two disulfide-linked subunits, p40, which is shared with IL-12 and a unique p19 subunit [9]. Similar to IL-12, only activated accessory cells such as monocytes, macrophages and DC concomitantly express both subunits to form the active cytokine [9]. Mice deficient for p19 can efficiently generate Th1 cells but fail to develop EAE after immunization with MOG [3, 10]. Considering these observations, it is evident that IL-23 and not IL-12 is a key player during the development of the autoimmune inflammation of the CNS. Initial studies suggested that IL-23 is involved in the generation of the newly described Th17 cells. However, other groups have shown that IL-23 alone does not influence the *de novo* differentiation of naïve T cells into Th17 cells *in vitro,* and it is only efficient in inducing the proliferation of committed IL-17 producing effector and memory T cells [11, 12]. Several groups have addressed the question as to which factors initiate Th17 polarization in naïve T cells. In contrast to Th1 and Th2 cells, the development of Th17 cells is not dependent of its respective effector cytokine (IL-17A). Instead, IL-6 and TGF-β, two cytokines with generally opposing effects, were found to direct the *de novo* generation of Th17 cells [11, 13, 14]. TGF-β induces the generation of Foxp3 expressing Treg, while addition of IL-6, a potent pro-inflammatory cytokine, deviates TGF-β-driven Treg toward Th17 polarization. Moreover, the expression of the transcription factor ROR-γt that has been shown to be critical for Th17 lineage determination is induced by the combination of TGF-β and IL-6 [15]. The mechanistic underpinnings of IL-23 function during clinical disease development and Th17 polarization is not yet completely understood, although some reports indicate that it is required for the survival and further expansion of already differentiated Th17 effector cells [16–18].

The receptor for IL-23 is a heterodimer composed of IL-12Rβ1 and a unique subunit, IL-23R [19], whereas the receptor for IL-12 consists of the shared IL-12β1 chain and the specific IL-12Rβ2 chain [20]. Thus, cells lacking IL-12Rβ1 cannot be engaged by either IL-12 or IL-23, while IL-12Rβ2-deficient cells are unresponsive to IL-12 but still responsive to IL-23. To trace IL-12 and IL-23-dependent behavior of T cells during EAE, we generated mixed BM-chimeric mice and found that in contrast to purified naïve T cells *in vitro*, encephalitogenic Th17 cell expansion *in vivo* is absolutely dependent on IL-23. Furthermore, loss of IL-23 signaling aborts the capacity of T cells to invade the CNS causing their accumulation in secondary lymphoid tissues.

Results and discussion

Construction of the model

IL-23 is a prerequisite for EAE development, but its role under pathological conditions remains elusive, due to the EAE resistance of mice lacking IL-12Rβ1$^{-/-}$, p40$^{-/-}$ and p19$^{-/-}$. In order to reveal IL-23-dependent T-cell behavior during EAE, we generated BM-chimeric mice (Fig. 1A). RAG1$^{-/-}$ mice were reconstituted with a mixture of either WT and IL-12Rβ1$^{-/-}$ BM cells or WT and IL-12Rβ2$^{-/-}$ BM cells. The gene-targeted cell population in the former chimera (IL-12/23R KO) is not responsive to IL-12 and IL-23, whereas the latter (IL-12R KO) is responsive to IL-23 but not to IL-12. The WT component of the graft is responsive to both cytokines and capable of initiating EAE permitting us to trace the behavior of the mutant T cells during inflammation. Each cell population carries a traceable congenic marker (CD45.1 = WT *versus* CD45.2 = IL-12Rβ1$^{-/-}$ or IL-12Rβ2$^{-/-}$) on the surface. After a recovery period, peripheral blood of chimeric animals was analyzed and the quantification of mature cell populations reflected the ratio (approximately 1:1) observed in the donor BM grafts (data not shown).

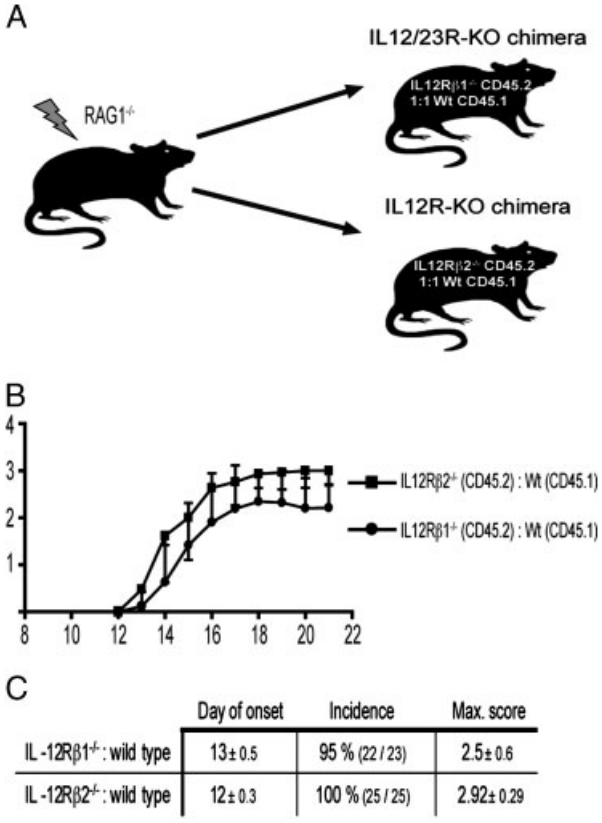

Figure 1. Generation of mixed BM-chimeras and EAE development in these animals. (A) Generation of the mixed BM-chimeras. Lethally irradiated RAG1$^{-/-}$ mice were reconstituted with either equal numbers of WT and IL-12Rβ1$^{-/-}$ BM cells (IL-12/23R KO chimera) or with equal numbers of WT and IL-12Rβ2$^{-/-}$ BM cells (IL-12R KO chimera). BM-donors are congenically different (CD45.1/2) and thus traceable. (B) Clinical scores after MOG_{35-55}-induced EAE (no significant difference between the two different groups). Error bars represent ± SD, data are representative of three independent experiments. (C) Detailed clinical development of EAE in IL-12/23R KO and IL-12R KO BM-chimeras.

To induce EAE, BM-chimeras were injected with MOG_{35-55} peptide emulsified in CFA without an additional injection of pertussis toxin. First signs of EAE were observed at 12 days post immunization (dpi) without significant differences between the two groups, in terms of disease incidence and disease severity (Fig. 1B and C) although IL-12R KO chimeras as expected showed a slightly more severe disease consistent with the hypersuscept-

ibility of the IL-12Rβ2$^{-/-}$ genotype. These mixed BM-chimeras allow the comparison of the behavior of WT and receptor-deficient cells in the same diseased animal which could not be studied in disease-resistant cytokine or cytokine receptor-deficient mice.

IL-23 affects the CNS-tropism of CD4$^+$ T cells but not systemic expansion and activation

In order to investigate the role of IL-23 on T cells during EAE, CNS-infiltrating cells were isolated from BM-chimeras and were analyzed by flow cytometry. Consistent with disease severity, massive infiltration of mononuclear cells was observed within the CNS of both groups. In the IL-12R KO chimeras we found a 1:1 ratio of WT and IL-12Rβ2$^{-/-}$ CD4$^+$ T cells infiltrating the CNS (Fig. 2A). In contrast, in the IL-12/23R KO chimeras we observed an overwhelming infiltration of IL-12/23 responsive WT CD4$^+$ T cells, whereas IL-12Rβ1$^{-/-}$ CD4$^+$ T cells failed to accumulate in the CNS (Fig. 2B). Absolute numbers of CNS-infiltrating T cells are provided in Supporting Information Fig. 1A. The failure to detect equal numbers of CNS-infiltrating IL-12Rβ1$^{-/-}$ CD4$^+$ T cells demonstrates the vital function of IL-23 in conferring encephalo-tropism of T cells, whereas the lack of IL-12-mediated signaling did not influence the capacity of T cells to infiltrate the CNS during EAE. In accordance with a recent report by McGeachy *et al.* the IL-12Rβ1-mediated impact on T-cell function is indeed mediated by IL-23R complex rather than additive effects of IL-23 and IL-12 [21]. To analyze the role of IL-12/23 or IL-12 signaling alone in the systemic immune compartment, spleen and LN cells were isolated at 16 dpi for cytofluorometric analysis from $MOG_{35–55}$ immunized BM-chimeras. As we observed previously, in the IL-12/23R KO chimeric animals where the number of IL-12Rβ1-deficient T cells infiltrating the CNS was drastically reduced, we detected their accumulation in both spleen and LN when compared with WT CD4$^+$ T cells in the same animal (Fig. 2C). At the same time in the IL-12R KO chimeric mice the ratio of peripheral IL-12Rβ2$^{-/-}$ CD4$^+$ T cells to WT CD4$^+$ T cells was unaffected (Fig. 2D). This finding indicates that the lack of IL-23R engagement does not interfere with the expansion of autoreactive T cells, but with the capacity to leave secondary lymphoid organs and to access their target tissue. Absolute numbers of T cells accumulating in secondary lymphoid organs are provided in Supporting Information Fig. 1A.

IL-12Rβ1 signaling is essential for the secretion of IL-17 by T cells *in vivo*

To address how IL-12 and IL-23 affect T-cell polarization *in vivo* during EAE, we analyzed the cytokine production of the infiltrating cell populations by intracellular cytokine staining and flow cytometry. IL-23R engagement on effector and memory T cells has been demonstrated to drive IL-17A secretion and Kebir *et al.* demonstrated that Th17 cells are able to cross the blood brain barrier and migrate to the CNS more efficiently than Th1 cells [16, 22]. Even though IL-17A itself plays only a minor pathogenic role during CNS-inflammation, it serves as a reliable marker for pathogenicity during EAE [4]. While the polarization

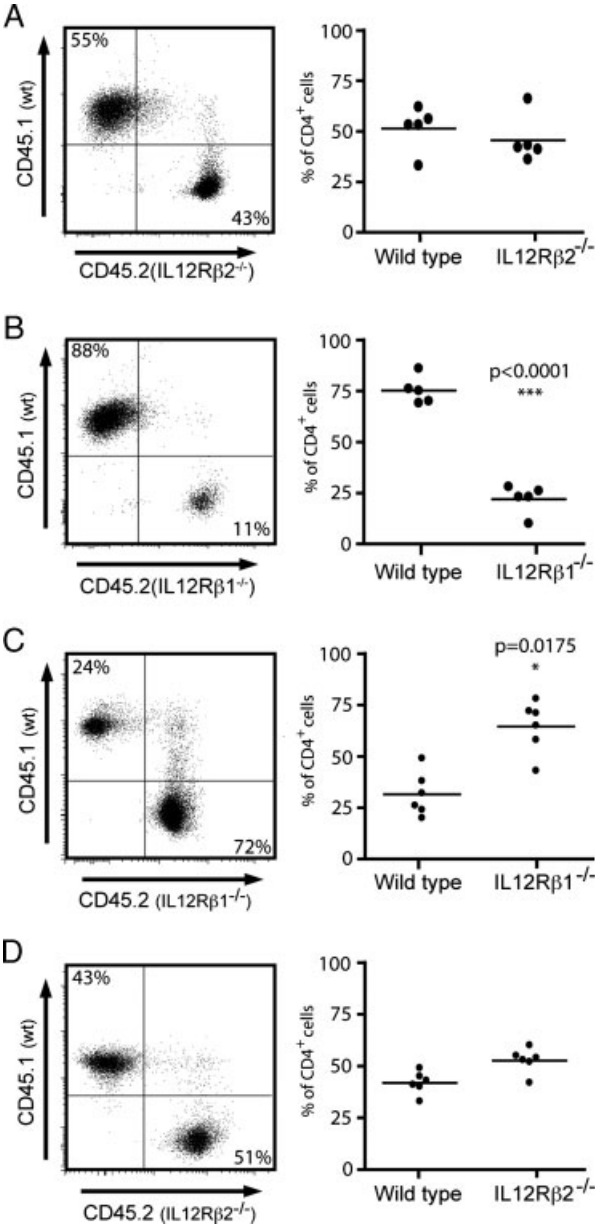

Figure 2. Absence of IL-12Rβ1 engagement but not IL-12 signaling results in the inability of T cells to invade the CNS leading to their accumulation in peripheral lymphoid organs. IL-12R KO and IL-12/23R KO chimeras were immunized with $MOG_{35–55}$/CFA. At peak disease (16 dpi), CNS-infiltrating leukocytes as well as cells from peripheral lympoid organs were isolated from either IL-12/23R KO chimeras or IL-12R KO chimeras and stained for congenic markers. The distribution of different cell populations was assessed by flow cytometry. Plots shown are gated on CD4$^+$ CNS-derived T cells. (A and B) CD4$^+$ T-cell compartment (CNS-infiltrating leukocytes) in the IL-12R KO or IL-12/23R KO chimeras, respectively. (C and D) CD4$^+$ T-cell compartment (spleen and LN) in the IL-12R KO or IL-12/23R KO chimeras, respectively. The panels to the right display individual percentages of all experiments combined. Data are representative of at least four independent experiments. Two-tailed Student's *t*-test was used to calculate *p*-values.

of highly purified naïve T cells toward a Th17 phenotype *in vitro* is IL-23 independent and relies on TGF-β and IL-6 exclusively [11, 13, 14], the impact of IL-23 on differentiation and expansion of Th17 cells *in vivo* is a matter of debate [17, 18, 23]. Cytokine analysis of the CNS-infiltrating cells revealed that WT $CD4^+$ T cells produced robust levels of IL-17A and IFN-γ, whereas CNS-infiltrating pathogenic IL-12Rβ2$^{-/-}$ $CD4^+$ T cells secreted higher levels of IL-17A than WT cells but failed to secrete IFN-γ (Fig. 3A and B). This is expected as IFN-γ inhibits the polarization toward the Th17 phenotype and likely acts in an autocrine and short-range paracrine fashion. Interestingly, IL-12Rβ1-deficient T helper cells, which infiltrate the CNS but do not respond to IL-12 or IL-23, fail to secrete both IL-17A and IFN-γ (Fig. 3C and D). McGeachy *et al.* just confirmed in a similar experimental design that IL-23R engagement is vital for Th17 polarization *in vivo* [21]. T cells lacking the IL-12Rβ2 on the other hand are not impaired in their capacity to invade the CNS, even though they fail to Th1 polarize. Absolute numbers of polarized Th1 and Th17 cells infiltrating the CNS are provided in Supporting Information Fig. 1B. Our data indicate that *in vivo* IL-23R engagement is critical for T cells to acquire encephalo-tropism and is essential for the production of IL-17A by CNS-infiltrating T cells.

Loss of IL-12Rβ1 alters the ratio Treg *versus* effector T cells in the CNS

TGF-β and IL-6 can polarize Th17 cells independent of IL-23, TGF-βR engagement enhances the generation of Treg. Foxp3 is the master transcription factor of Treg and is expressed by the precursor to both Th17 and Treg. IL-6 induces the induction of ROR-γt and Runx1, which are thought to be the main switch that re-directs T-cell differentiation from the regulatory phenotype toward the effector Th17 phenotype[24]. To determine whether IL-23 plays any role in Treg or Th17 lineage commitment during inflammation, we assessed the number of $Foxp3^+$ regulatory $CD4^+$ T cells among the infiltrating leukocytes by cytofluorometrical intracellular cytokine analysis. Even though the total number of effector T cells from the IL-12Rβ1$^{-/-}$ population is drastically reduced, within this population, we observed the preferential accumulation of Foxp3 positive cells (Fig. 3E and F). None of the other populations (IL-12Rβ2$^{-/-}$ or WT) revealed an elevated percentage of $Foxp3^+$ cells. Based on this observation, we speculate that IL-23 affects the CNS-tropism and encephalitogenicity of effector T-cell while Tregs are unaffected. It is feasible that without the trophic support by IL-23, effector T cells could

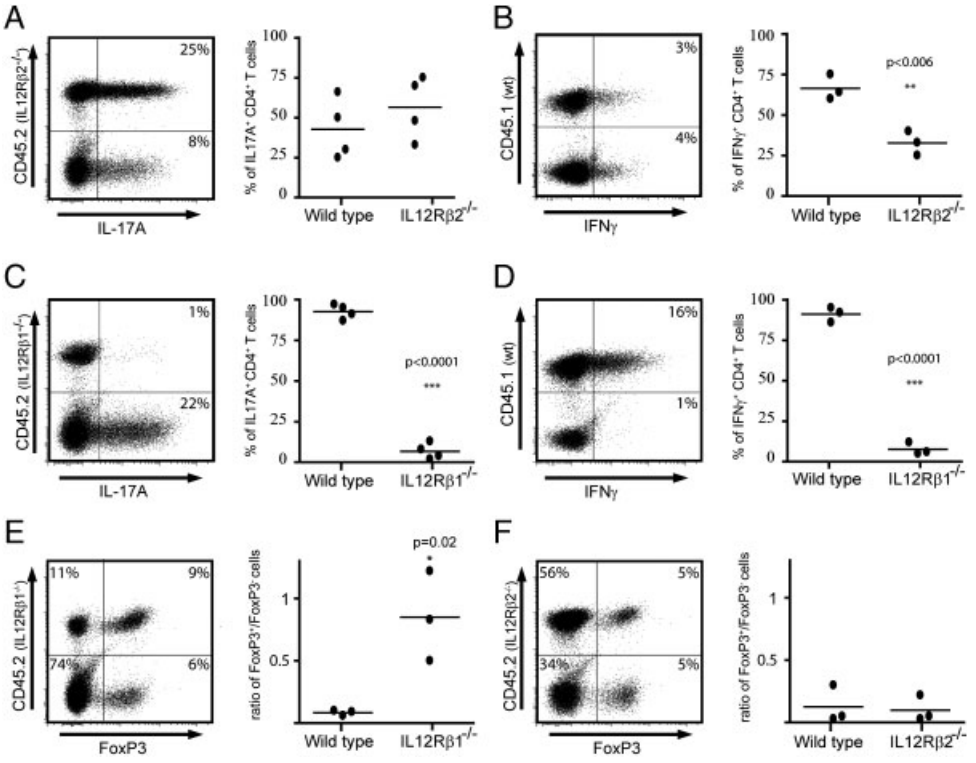

Figure 3. Loss of IL-12Rβ1 engagement leads to decreased production of proinflammatory cytokines by $CD4^+$ T cells and preferential accumulation of $Foxp3^+$ $CD4^+$ T cells in the inflamed CNS. Expression of IL-17A and IFN-β was measured by intracellular cytokine staining. Dot plots are gated on $CD4^+$ CNS-derived T cells. Percentages of IL-$17A^+$ and IFN-β^+ cells are given in the quadrant corners. (A and B) Cytokine production by CNS-infiltrating $CD4^+$ T cells derived from the IL-12R KO BM-chimeras, IL-17A and IFN-β, respectively. (C and D) IL-17A and IFN-β secretion by CNS-infiltrating $CD4^+$ T cells derived from IL-12/23R KO BM-chimeras. The expression of Foxp3 was measured by intracellular cytokine staining. Dot plots are gated on $CD4^+$ CNS-derived T cells. (E) Foxp3 expression in the IL-12/23R KO BM-chimeras. (F) Foxp3 expression in the IL-12R KO BM-chimeras. Data are representative of at least three independent experiments. The panels to the right display individual percentages of all experiments combined. Two-tailed Student's *t*-test was used to calculate *p*-values.

undergo apoptosis upon entering the CNS. We did not find any increase in AnnexinV$^+$ cells extracted from the CNS of mice suffering from EAE. In addition, given that we observed a clear expansion and accumulation of IL-12Rβ1$^{-/-}$ T cells in secondary lymphoid organs, we conclude that IL-23 affects CNS-tropism, rather than longevity or apoptosis.

Concluding remarks

The role and impact of IL-23 *versus* IL-12 on autoimmune T cells remains a subject of intense debate. While *in vitro* generated Th1 and Th17 cells can both elicit inflammatory CNS lesions in EAE [25], the factors that control Th1 and Th17 polarization, impact on more than just the T-cell cytokine profile. Th17 polarization was demonstrated to be independent of IL-23, but the reports dismissing IL-23 in favor of TGF-β and IL-6 as exclusive Th17 promoting factors relied on the use of highly purified CD4$^+$ T cells and *in vitro* expansion [14]. In this report here, we confirm that *in vivo* and during EAE, IL-23 is absolutely critical for Th17 polarization and the acquisition of encephalitogenicity [21]. In contrast to IL-23, Th17 signature cytokines are not essential for the development of CNS autoimmunity [4, 5]. While IL-17 clearly affects the CNS endothelium, loss of IL-17A and F only leads to reduced inflammation, but not in complete EAE resistance [4]. Thus IL-23-mediated effects on T-cell pathogenicity is a feature far beyond T-cell polarization. Here, we demonstrate that IL-23 not only promotes Th17 polarization but also and more critically T-cell-CNS-tropism while loss of IL-12 signaling and the polarization toward a Th1 phenotype is not essential for the tissue infiltrating capacity of auto-reactive T cells.

Materials and methods

Mice

C57BL/6, IL-12Rβ1$^{-/-}$, IL-12Rβ2$^{-/-}$ and RAG1$^{-/-}$ mice were purchased from Jackson laboratories (Bar Harbour, Maine) and bred in house under specific pathogen-free conditions. Animal experiments were approved by the Swiss Veterinary Office (13/2006; Zurich, Switzerland) and performed according to federal and institutional guidelines.

To generate BM-chimeras, RAG1$^{-/-}$ mice were lethally irradiated with a split-dose of 1100 rad. Donor animals were euthanized with CO_2 and bones (fore- and hind legs, hips) were flushed with sterile PBS to obtain BM stem cells. Mutant and WT BM with the respective congenic marker were mixed at a 1:1 ratio. The mixture was verified by flow cytometry with anti-CD45.1 (A20) and anti-CD45.2 (104) mAb (BD Pharmingen). In total, 2×10^7 cells were injected i.v. *per* mouse and to prevent bacterial infection 0.2% BORGAL was added for 2 wk to the drinking water.

Induction of EAE

Six to eight weeks after reconstitution BM-chimeras were immunized subcutaneously with 200 μg of MOG_{35-55}-peptide emulsified in CFA into the flank. Clinical disease was scored daily as follows; 0,– no clinical disease; 1, limp tail; 2, impaired righting reflex; 3, hind limb paralysis; 4, moribund; 5, dead.

Fluorocytometric analysis of splenocytes and mononuclear cells from the CNS

Extraction of mononucleated cells from inflamed CNS tissue was performed as described previously [26]. Mononucleated cells were stimulated in RPMI 1640 containing 10% FBS (both from Invitrogen) supplemented with PMA (50ng/mL) ionomycin (500ng/mL) and BD GolgiPlug™ (1 μL/mL) for 5 h at 37°C. Fluorocytometric analysis of surface marker expression was performed as described [26]. Intracellular cytokine staining was performed with the Cytofix/Cytoperm™ Plus Kit (BD Bioscience) according to the manufacturer's directions. The following antibodies were used: anti-IL-17A (TC11-18H10), anti-IFN-γ(XMG1.2) (BD Pharmingen), anti-Foxp3 (FJK-16s) (eBioscience).

Statistical analysis

The significance of the difference in percentages/ratios of different cell populations was evaluated using a two-tailed Student's *t*-test. *p*-Values of less than 0.05 were considered significant.

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Abbreviation: **dpi:** days post immunization

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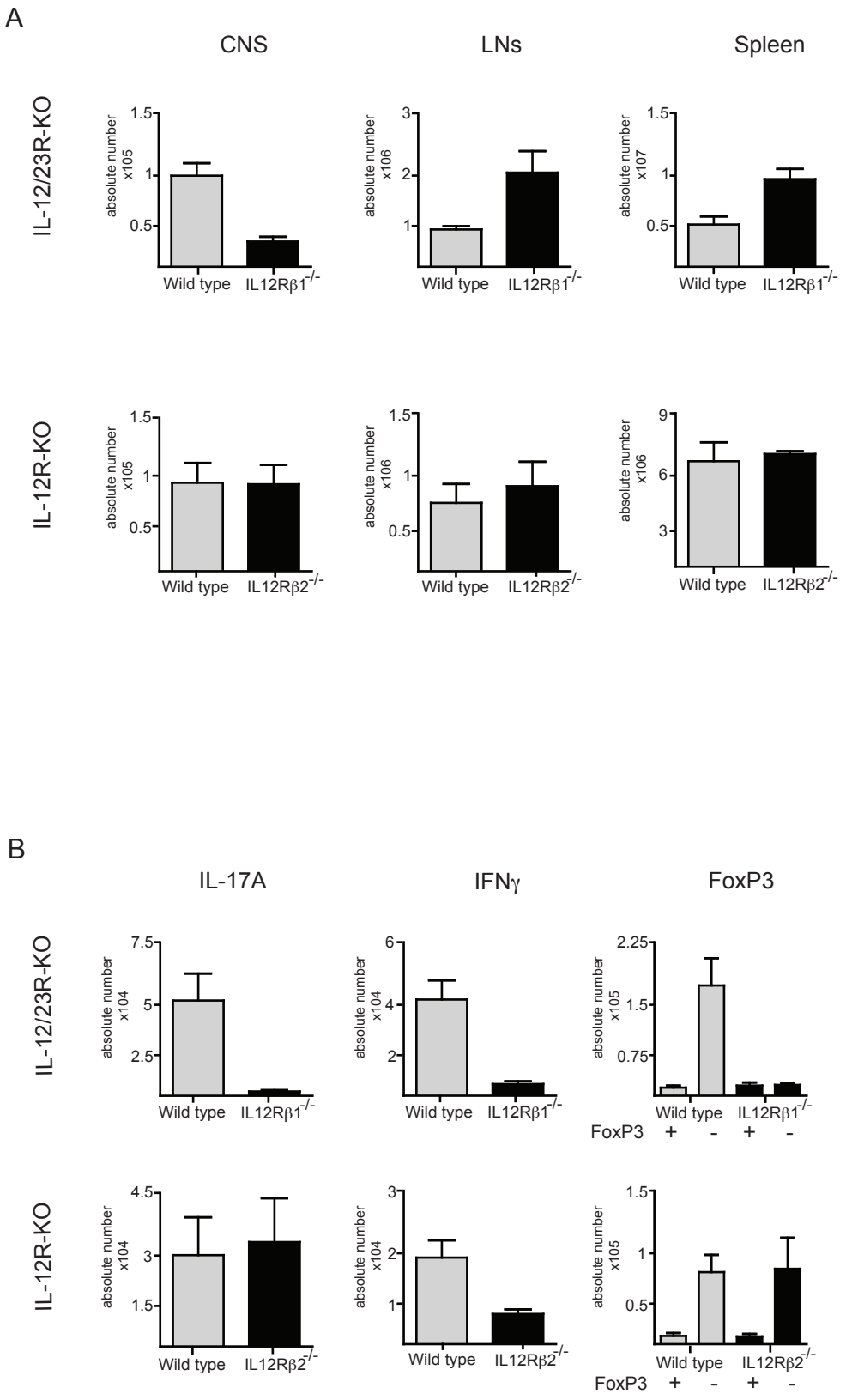
Supporting Information Figure 1



Discussion

In 2002 our lab started to experimentally address the relevance of IL-12 and -23 in EAE, asking how IL-23 renders TH cells pathogenic. This question became a focus of research in many labs over the years, but has not yet been resolved. To gain insight into the transcriptional changes underlying the pathogenic effector responses, which are triggered in TH cells, we devised two reciprocal approaches for whole genome transcriptomics. In the analysis of the IL-23 inducible genes we screened for factors of which upregulation was missing in *in vivo* primed effector TH cells specifically due to loss of IL-23 signalling [KREYMBORG K ET AL. 2007]. Only 5 genes met the stringent criteria, four of which where effector cytokines of TH17 cells, IL-22, -17A, -17F and GM-CSF. This set of data has been the basis of our interest in the actual relevance of IL-23 driven effector pathways in organ-specific autoimmunity. One aspect, the impact of IL-17A, IL-17F and IL-22 on EAE development, is presented in this study.

TH17 effector function

There has been substantial confusion about the true nature of the role of IL-17A in autoimmune neuroinflammation. In the late nineties, research was still relatively slow on the newly discovered cytokine. Nevertheless, IL-17A was already noticed as a primarily T cell secreted cytokine with an unusual affiliation with effector TH cells that were not TH2, probably not TH1 and obviously not suppressive, since IL-17A was known to have a high proinflammatory potential [FOSSIEZ F ET AL. 1996, AARVAK T ET AL. 1999, CHABAUD M ET AL. 1999]. It was deduced that those T cells should be some type of alternative TH1 also nourished by the finding that it exerts synergistic effects with IFNγ on keratinocytes [TEUNISSEN MB ET AL. 1998]. Until 2003 it was revealed that IL-17A induces the recruitment of neutrophils and is important in bacterial infections of lung and gut. However, the main interest in the cytokine lay in the area of autoimmune research. Its presence and activity has already at that time been connected to autoimmune inflammation in the skin [TEUNISSEN MB ET AL. 1998], the CNS [MATUSEVICIUS D ET AL. 1999] and most of all in the joints [KOTAKE S ET AL. 1999]. In 2003 the revelation that IL-23 and not IL-12 is essential for the development of organ-specific autoimmunity, in this case EAE, and that IL-23 stimulates, while IL-12 counteracts, the generation of IL-17A by T cells formed the notion that the IL-23/IL-17A axis of T cell mediated immunity is the pathogenic effector type that was formerly believed to be the IL-12/IFNγ axis [GRAN B ET AL. 2002, BECHER B ET AL. 2002, AGGARWAL S ET AL. 2003]. The idea was born but had to be proven. HOFSTETTER ET AL. followed up on the threads that already connected IL-17A with autoimmune neuroinflammation and conducted a simple and concise study on one of the most critical questions, the potential in the therapeutic use of antagonising IL-17A signalling in EAE. The authors used two parallel approaches, anti-IL-17A antibodies (R&D MAB421) and an IL-17RA-Fc fusion-protein potentially ablating both

IL-17A and IL-17F signalling. The neutralising capacity of the soluble receptor, however, was not shown. The generated results had the clear massage that neither of the treatments led to a dramatic amelioration of disease, whereas only a mild phenotype was consistently found. Despite the qualities of the study, HOFSTETTER struggled to interest publishers for it, mainly because the results were just not positive enough. The rather sobering results also seem to had come at an inopportune time at which IL-17A was pushed out of the shadow of indifference into the spotlight by the publications of the groups of WEAVER and DONG. Those reports presented data supporting the conclusion that IL-17A marks a new lineage of T_H effector cells [PARK H ET AL. 2005, HARRINGTON LE ET AL. 2005]. LANGRISH ET AL. from DNAX Research Inc published at the same time that these T_H17 cells are the true pathogenic T_H cell type shown by adoptive transfer of primed T_H17 cell in EAE. Their encephalitogenic potential was dominantly mediated by the T_H17 effector cytokine IL-17A, since application of neutralising antibodies showed a strong amelioration of EAE [LANGRISH CL ET AL. 2005]. The anti-IL-17A antibody (DNAX/BD 18H10) was only given once at the day of disease onset as compared to three times (before, at and after) disease onset in the HOFSTETTER study [LANGRISH CL ET AL. 2005, HOFSTETTER HH ET AL. 2005]. The stronger phenotype published higher and the difference in visibility in the public domain seems to have had a great influence on the future of IL-17A research. The interpretations of the data produced on IL-17A in the following years appeared to have been significantly biased towards the suggested critical role of IL-17A in EAE taken mainly from the LANGRISH study, although opposed by the HOFSTETTER study.

A year later, in 2006, the group of IWAKURA published the strongly awaited loss-of-function study using their IL-17A deficient mouse strain to complement and consolidate the divergent results when blocking antibody regimes were applied. Considering the conclusion that is discussed in the LANGRISH paper, that loss of IL-17A should render mice resistant to EAE, the results of IWAKURA'S group were clear; upon active induction of EAE all mice developed disease [KOMIYAMA Y ET AL. 2006]. However, the course of disease was different. IL-17A deficiency led to a prominent delay in the day of onset although the subsequent succession of disease stages was as rapid as in wt mice. Unfortunately no table was presented with the mean of maximum disease severity and incidence of all experiments that were conducted. The severity was described as being milder at peak disease for the IL-$17A^{null}$ mutant mice. The main phenotype, however, was an extraordinary recovery of the IL-17A deficient mice at around 10 days past disease onset. Interestingly, when EAE was induced without the application of PT this severe difference in the recovery of the IL-17A mice was lost. The interpretation of this possible difference at late time points of the disease is difficult, since this acute EAE model is inappropriate to address a cytokines role in chronic inflammation. It is possible that the neuronal damage in the presence of IL-17A was of a different quality than the one elicited in the deficiency model which only became visible in the long term consequences. In general it is common believe that the T_H17/IL-17 axis is particularly active in chronic inflammatory settings. This, however, is far from being proven.

The most robust effector responses that are IL-17A dependent are found in acute infection models where IL-17A mediates critical cross-talk of innate and adaptive immunity. Apart from T cells, innate sources of IL-17A contribute greatly in these acute stages (e.g. γδT cells, neutrophils). Also in EAE the dominant TH17 activity is found at the acute stages, during early and peak disease [REBOLDI A ET AL. 2009]. A similar model of organ-specific autoimmunity also concerning an immuno-privileged area is experimental autoimmune uveoretinitis (EAU), a common murine model for human endogenous uveitis such as Vogt–Koyanagi–Harada disease and Behcet's disease. In many mechanistic aspects, e.g. dependence on IL-23/STAT3 signalling, EAU proved very comparable to EAE [AVICHEZER D ET AL. 2000]. In EAU TH17 ingress into the eyes mark the acute phases of the disease whereas post peak disease TH1 cells clearly dominate the scene. Although this is often interpreted as prove that TH17 cells are pathogenic and TH1 facilitate recovery, this categorisation is at odds with TH1 cells being fully pathogenic in passively induced disease [AMADI-OBI A ET AL. 2007]. The involvement of TH17 cells in chronic aspects of diseases might be indirect by means of their strong proinflammatory influence during acute inflammation which could lead to long lasting tissue damage that could play a role in chronification.

In addition to the active induction model KOMIYAMA ET AL. adoptively transferred primed IL-17A^{null} T cells into wt mice to allow an effector response in a wt context. Also here, the IL-17^{null} mutants performed poorer, even more pronounced that in the active EAE model. Whether this difference in active versus passive EAE has a biological reason or whether it is due to accumulated uncertainties in the adoptive transfer model remains unclear. In early 2008 the group of YOSHIDA investigated the impact of loss of IL-17A for the development of EAU. They found that upon active immunisation with retinal antigens plus CFA/PT there was no difference in the development of the disease until peak disease. Similar to the situation in EAE, the recovery under IL-17A deficient conditions was a bit faster and slightly more pronounced [YOSHIMURA T ET AL. 2008].

Unperturbed by the actual data available, however, the notion that IL-17A is essential for disease development remained and was repeated in writing in major publications on the topic of IL-17A bringing forth the possibility that conclusive evidence exists but is just not available in the public domain. Consequently by a way of scientific gossip mice lacking IL-17A were said to be resistant to EAE and along with the overwhelming amount of correlative data connecting IL-17A with autoimmunity, the cytokine was conventionalised to be the master effector cytokine in organ-specific autoimmunity [LANGRISH CL ET AL. 2005, IVANOV II ET AL. 2006].

The overall massage from the publications so far on the role of IL-17A in EAE is that, IL-17A, unlike IL-23 is not essential for the development of EAE. Since the strongest general phenotype found in IL-23p19 deficient mice was the absence of a TH17 response and the fact that TH17 cells are highly pathogenic in the adoptive transfer model of EAE encephalitogenicity could also be mediated through one of the other TH17 effector cytokine.

The role of IL-17A and IL-17F in EAE

To test the hypothesis, that TH17 exert their encephalitogenic potential through the secretion of their three signature cytokines we performed a comprehensive study based on the respective genetic deficiency models. Since, despite previous studies on that matter, there was no true consensus on the importance of IL-17A for the development of EAE we started our study with the IL-17A deficient mouse strain and complemented the loss-of-function study with a gain-of-function analysis to generate a conclusive picture. For the gain-of-function study we generated a mouse strain overexpressing IL-17A and eGFP exclusively in T cells. Already in 1998 and 2000 the group of KOLLS attempted to overexpress IL-17A by systemic application of an IL-17A encoding adenovirus. Ectopic expression resulted in a dramatic mobilisation of neutrophils via G-CSF [SCHWARZENBERGER P ET AL. 1998 & 2000]. Since resting lymphocytes have a generally low transcription rate, strong ectopic secretion of IL-17A in our model was only observed in activated T cell, which focused overexpression to the sites and time of T cell priming and effector function. Accordingly, systemic alterations at steady-state were only visible in a mild granulocytosis as compared to a massive granulocyte increase in the total-overexpresser mouse. Once the mice were immunised, IL-17A overexpression accelerated resulting in 60 percent of TH cells in the CNS of mice at peak disease expressing IL-17A as compared to 16 percent in the control group. This massive local increase in IL-17A expression, however, had no impact on the course of disease clearly contradicting the anticipated exacerbation of disease. The lack of a clinical phenotype in the gain-of-function study does not suggest a pivotal role of IL-17A in the autoimmune inflammation but also does not exclude the possibility of it. A lot of new insight has been gained over the last few years on the signalling of IL-17A but especially the early signalling events and possible feed back loops are still unknown. IL-17A signalling could be saturated already at physiological concentrations of the ligand. Since IL-17A signalling shares many features in terms of signalling elements and its proinflammatory capacity with the TLR family it could similarly contain a negative feed back mechanism that at a certain signalling threshold results in a transient state of refractoriness. In the case of the TLR system, this protects the organism of excessive inflammation that could otherwise lead to septic shock. To be conclusive, the IL-17A^{null} mutant strain was analysed in actively induced EAE. The analysis was performed with a relatively large number of mice to allow in depth statistical analysis. Our results confirm the overall findings of the IWAKURA study [KOMIYAMA Y ET AL. 2006] that IL-17A is redundant in the development of EAE, since there is no significant difference in the incidence of disease. We also confirm a statistically significant delay of the day of disease onset, although in our hands this difference was only about one day. The average maximum severity of the mice was mildly decreased but not statistically significant. All IL-17A^{null} mice developed disease and with clinical scores comparable to the wt controls but then recovered faster and slightly more than the controls. We could not confirm the extraordinary recovery observed by KOMIYAMA about 10 days past disease onset in the comparable EAE study with application of PT but not in their study without PT. This

difference between the two studies, the one from IWAKURA'S group and ours, is too striking to be explained by differences due to binning effects in the individual scoring procedures of the two labs. EAE, like many other inflammatory animal models, has the disadvantage to be sensitive to environmental parameters that are not constant throughout different animal housing facilities, e.g. microflora. Those parameters influence the steady-state condition of the animal's immune system significantly and can result in partially different outcomes for the identical experimental procedure. Slight differences in the mouse genetic background can also account for such discrepancies. In the further analysis of the IL-17A deficient mice it became apparent that loss of IL-17A expression by *in vivo* primed T_H cells leads to a possibly compensatory upregulation of IL-17F production when restimulated *in vitro*. The assumption of a compensatory role of IL-17F in IL-17A deficient mice conceivable, since IL-17A and -17F are closely related and share an exceptionally similar pattern of expression and function. Their signalling is facilitated by the same receptor subunits and transduced into a similar transcriptional activity pattern. At the time of the study it has not been clearly shown whether IL-17F is expressed significantly in the lesioned CNS of EAE mice alongside with IL-17A. We confirmed the expression at peak disease and showed that IL-17F is typically co-produced with IL-17A in T_H17 cells. To address the hypothesis that IL-17F compensates for the loss of IL-17A in EAE we first investigated whether IL-17F itself has a critical role in EAE. For this we generated an IL-17F^{null} mouse strain. For our study it was not fully backcrossed on the C57BL/6 genetic background, which was compensated by the use of littermate controls and a relatively high number of experimental mice. Il-17F^{null} mutants are healthy and capable of normal T_H cell priming, polarisation and expansion. To our great surprise IL-17F deficiency neither had any clinical nor histological nor cellular phenotype in EAE. Our results confirm the results obtained with a different IL-17F^{null} mutant mouse generated in the lab of DONG that was published slightly earlier than our work [YANG XO ET AL. 2008]. Although our finding was "negative" and was disregarded by the editorial boards of some major scientific journals, they brought up a conceptional change on the view on the role of the two IL-17 brothers. With ours and DONG'S studies of the IL-17F^{null} mutant mice under inflammatory conditions, a severe difference in the role of IL-17A and -17F has been observed. The reasons behind this, however, are still not understood. Since the early times of IL-17 research it has been frequently shown that in many *in vitro* settings the proinflammatory trigger of IL-17A is stronger than of IL-17F signalling. Hence, the proinflammatory capacity of the two cytokines might be different *in vivo* as well. Intriguingly, two studies on this topic measuring the proinflammatory response of human colonic or epidermal tissue revealed an obvious difference between IL-17A and -17F that was not expected. The colon derived cells responded stronger to IL-17A while the keratinocytes responded stronger to IL-17F suggesting a tissue depending role of the two cytokines. Since then, more evidences has been collected proving that IL-17F is not just a less potent IL-17A. In IBD mouse models there is good evidence from loss-of-function and gain-of-function studies that IL-17A serves a protective function, whereas IL-17F was found to be pathogenic through the induction of proinflammatory factors that lead to exacerbation of

disease [YANG XO ET AL. 2008, O'CONNOR W JR ET AL. 2009]. In inflammatory responses in the skin the situation is less clear. Our lab is currently investigating on the specific role of IL-17F *versus* IL-17A in psoriatic plaque formation. In allergic inflammation in the lung YANG ET AL. indicated that IL-17F but not IL-17A plays an important role in mediating innate responses to allergens in an IL-17RA-dependent manner [YANG XO ET AL. 2008]. In models of rheumatoid arthritis the observations are similar to the ones we described for EAE; while IL-17A has a comparatively strong effect on the severity of disease, IL-17F does not show the same potency [ISHIGAME H ET AL. 2009, reviewed in LUBBERTS E 2010]. It seems to be a common trait of T_H cell mediated autoimmune diseases that IL-17F is negligible, while IL-17A is more or less involved, depending on the organ.

The interesting question which now has to be addressed more thoroughly is, how IL-17A and -17F exert their different effector responses. One factor seems to be the source and kinetics of their individual secretion. IL-17A and -17F are known to be co-produced by activated αβ and γδT cells but not necessarily at the same rate at the same time. In inflammatory models inflicting other organs than the CNS there is first data accumulating suggesting differential expression. In the colon during *Citrobacter rodentium* infection, the two cytokines play differential roles with IL-17F being more efficacious. It was found that the two cytokines were preferentially expressed by different cell population and also at different sites of the digestive tract. IL-17A was mostly expressed by T cells in the small intestine while IL-17F was more expressed by non-lymphocytes in the colon [ISHIGAME H ET AL. 2009]. However, in *in vitro* polarisation assays as well as in the CNS of EAE mice, the expression appears to be mostly tied to each other in T_H17 cells [CROXFORD AL ET AL. 2009, HAAK ET AL. 2009]. But also cells of the innate compartment infiltrate the CNS in EAE and contribute to the amount of the secreted "T_H17 cytokines". The major innate cell population in EAE are probably γδT cells. The little amount of data available does not allow judgement on where and when the individual cytokines are secreted by those cells. The parameter which is probably the most crucial in the divergent IL-17A and -17F signalling responses are differences in the receptor complex composition which determines the respective responsiveness to either cytokine and the molecular responses they elicit. It is speculated that the differential expression rate of IL-17RA, -17RC and possibly other receptor units result in differential stoichiometric receptor complex arrangements that mediate IL-17A and -17F binding differently, leading to a differential cellular responses. In this regard the observed tissue dependent differences of IL-17RA and -17RC expression might play an essential role. Another possibility especially in tissues like the brain, where IL-17RC expression is scarce, is the involvement of the proposed agonistic IL-17RC soluble receptor isoform in *trans*-signalling, comparable with the IL-6 or IL-15 system. In mice, unlike in humans, IL-17RC only has affinity to IL-17F, nevertheless, in all experimental systems where this has been explicitly tested so far IL-17RC was a requirement for signalling of IL-17A and -17F. This rule, however, seem to not completely apply, since recently it has been found that IL-17A can signal on T_H cells that only express measurable amounts of IL-17RA [O'CONNOR

W JR ET AL. 2009]. A similar effect has not been observed so far with IL-17F, suggesting that in the complete absence of IL-17RC only IL-17A can signal at least in TH cells [ISHIGAME H ET AL. 2009]. One issue that is potentially complicating matters when comparing IL-17A and -17F is the existence of heterodimers. From the intermediate bioactivity of the heterodimer it might be concluded that the function of IL-17F can be in downregulating the IL-17A signalling efficacy [WRIGHT JF ET AL. 2007 & 2008]. This would suggest that the absence of IL-17F in autoimmune inflammation would result in an increase of IL-17A homodimers and a possible increase in disease severity. EAE, the model of our study, however, would be the wrong model to address this point, since we have shown in IL-17A overexpressing mice that an increase of IL-17A availability does not lead to an alteration of the course of disease. On the other hand, to rule out a compensatory role of IL-17F in the IL-17A deficiency model, we treated IL-17F deficient mice with a neutralising anti-IL-17A antibody to be able to analyse EAE development in the absence of both cytokines. Our study is the first to address the issue of a possible compensatory effect between the two IL-17 cytokines by generating a double deficient situation. It is not possible to generate this by mere interbreeding of the single mutants due to the close proximity of the two gene loci. The decision to use the constellation where anti-IL-17A is applied was due to the fact that anti-IL-17A treatment is established and published in a number of papers whereas anti-IL-17F treatment has not been equally attempted and there are fewer antibodies available. Our antibody treatment regime resembles the one applied by LANGRISH ET AL. but with four times as much antibody distributed over three more days of treatment [LANGRISH CL ET AL. 2005]. The results of our experiments were cogent. The double deficient mice showed the same mild phenotype in EAE as the IL-17A^{null} mutant mice did. Hence, we are confident to conclude from our single and double deficient models that there is no compensatory or additive effect between IL-17A and-17F in EAE. In the last year, complementary data has been presented by other groups in models of organ-specific autoimmune diseases. The group of IWAKURA presented roughly at the same time of our publication the first study on a double targeted mouse strain that lacks IL-17A and IL-17F [ISHIGAME H ET AL. 2009]. They also investigate on the possible additive effects of the two cytokines in a variety of disease models. Their study adds to ours that also in CIA and in the spontaneous autoimmunity model of IL-1 receptor antagonist deficient mice there are no conceivable additive effects between the cytokines and only the loss of IL-17A has a considerable phenotype depending on the disease model.

The role of IL-22 in EAE

In our study we stressed the extraordinary dependency of IL-22 on IL-23. In our gene array analysis in pursue of the factors correlating with IL-23 induced encephalitogenicity, IL-22 was the cytokine most upregulated in lymphocytes. In agreement with LIANG ET AL. we found in this study IL-22 to be highly expressed by a certain moiety of the TH17 cells and discovered it as a marker that correlated well with encephalitogenicity along with IL-17A, -

17F and IFNγ which were upregulated in the same inflammatory context [LIANG SC ET AL. 2006, KREYMBORG K ET AL. 2007]. As for IL-17A, -17F and IL-21, IL-22 was found in a recent study by SUTTON ET AL. to be also expressed by γδT cells in the lesion CNS of EAE mice [SUTTON CE ET AL. 2009]. The amount of IL-22 produced by these innate cells and the role it plays in the inflamed CNS has not been reported to date. To answer the question about the role of IL-22 of any source for EAE development, we generated an IL-22^{null} mutant mouse strain by partial deletion of exon 1 to 3. In humans as well as in BALB/c and DBA/2 inbred mouse strains IL-22 is a single copy gene whereas in C57BL/6, FVB and 129 mice the IL-22 (syn. IL-22α) coding gene has a duplicate, termed IL-22β. IL-22β shows 98% nucleotide identity in the coding region while differing by a deletion of 658bp in the 3'UTR and putative promoter region as well as an insertion of 385bp intron 4. Due to the loss of promoter structure it is suggested to be inactive [DUMOUTIER L ET AL. 2000]. Our gene-targeting only affected the functional *il22a* gene. Thus we verified the absence of transcription of IL-22 by PCR with primers amplifying IL-22α and -22β. On the protein level the absence of IL-22 translation was tested by ELISA. In agreement with RENAULD'S group we confirmed the absence of IL-22β transcription in the C57BL/6 mouse strain. With our IL-22^{null} mutant being confirmed to be completely deficient of IL-22 we proceeded to address the causal link between the observed increase of IL-22 expression and EAE lesioned CNS. To our great surprise lack of IL-22 did not impact at all on the development of EAE in any clinical, histological or cellular aspect. In general IL-22 effector function seems to be tightly restricted to the relatively few organs and tissues that express the IL-22R1 subunit of the receptor complex. The R2 subunit, IL-10R2 is basically ubiquitously expressed. The prime organs where the receptor complex is dominantly expressed are also the organs where it has been shown that IL-22 impacts vitally on an inflammatory response, which are the skin, the digestive and the respiratory tract. In that respect our finding on the impact of IL-22 in the CNS is not surprising, since its receptor is generally not detectible there [KOTENKO SV ET AL. 2000, WOLK K ET AL. 2004]. However, it is not necessarily a prerequisite that the receptor is expressed by a large cellular mass in the organ if the expression can instead be located to a minor but crucial cell population for the generation of an inflammatory response. In a collaborative effort with the lab of PRAT we discovered first evidence of the expression of IL-22R and IL-17R on a subset of human BBB epithelial cells in primary cell culture and *in situ* at MS lesions [KEBIR H & NINCHEN ET AL. 2007]. We were able to show *in vitro* that IL-22 and IL-17A signalling in those cells led to an increase in epithelial permeability due to a weakening of tight junctions allowing solute and cellular transfer. A comparable structural change in the integrity of the BBB has been correlated to vessels at highly infiltrated MS lesions [WOSIK K ET AL. 2007]. However, there is no certainty about the necessity of these BBB changes for the course of the disease. Moreover, it has not yet been resolved if these findings also apply to the mouse system. Granted that BBB permeability is also affected by these cytokines in the mouse, our *in vivo* results on the development of EAE in the cytokine deficient models would rather suggest that this ability of IL-22 and IL-17A is redundant. Although to ultimately exclude reciprocal compensatory effects in the single cytokine

deficiency models, EAE development would need to be addressed in an IL-22/IL-17A double deficient mouse strain.

While IL-22 overexpression has been also correlated with the synovium of rheumatoid arthritis patients and the site of psoriatic plaque formation, the only promising experimental results have been found in respect to keratinocyte activation and dysregulation in inflammation of the skin [IKEUCHI H ET AL. 2005, ZHENG Y ET AL. 2007, WOLK K ET AL. 2006 & 2009]. Although GEBOES ET AL. reported a pathogenic effect of IL-22 in CIA, this effect was in fact fairly mild and does to our understanding not suggest a major role of IL-22 in this model of joint inflammation [GEBOES L ET AL. 2009]. Taken together it seem fair to conclude that IL-22, partially due to the fact that it signals to sessile stromal cells and partially due to the restrictiveness of IL-22R expression exert its most dominant functions only a few specific organs like the skin, the lung, the liver and the gut, but not the CNS or the joints. Hence, its role seems to be more defined by the location of the immune response as opposed to the cause of the response provided that its secretion is generally triggered in the respective context.

It should be noted that while IL-22 has been found to mediate crucial immunomodulatory functions in skin, liver, lung and gut inflammation, these functions largely depend on IL-23 dependent production of IL-22 by innate cells rather than on the dominant action of T_H17 cells [ZENEWICZ LA ET AL. 2007, ZHENG Y ET AL. 2007 & 2008, AUJLA SJ ET AL. 2008].

Implications for the role of T_H17 cells in autoimmunity

In order to manipulate the immune response in autoimmune diseases in a way to prevent or stop chronic inflammation directed to self it is the primary goal to identify and characterise the cell type that is responsible for the pathogenicity. MS and EAE are T cell driven diseases, yet which of the effector types is to blame and ultimately to target in a therapeutic approach is still a matter of debate. In the dispute about the role of T_H1 *versus* T_H17 effector type the most basic studies were performed on the mouse strains completely lacking either effector type. Closest to that state are the respective "master TF" deficient models. T-bet^{null} mice lack the T_H1 lineage and were found to be EAE resistant [BETTELLI E ET AL. 2004]. While this could be construed to strengthen the notion that T_H1 cells are the encephalitogenic perpetrators in EAE, the lab of GLIMCHER and others demonstrated that T-bet deficiency affects not only T_H1 polarization but also lesions many other relevant cellular processes, like activity of APC [WANG J ET AL. 2006]. A similar problem was encountered in the (RORgt/RORa)^{null} double mutant mice that lack the T_H17 lineage. Like T-bet^{null} mutants those mice were found to be resistant to EAE but they also comprise a number of other severe cellular defects, like deficits in SLT organisation and thymopoiesis (cf. "Introduction: T_H17 cells") [YANG XO ET AL. 2008].

One way to potentially study the impact of a specific effector type is by the use of gene targeted mouse models in which T cell mobility was selectively impaired. Most interesting were the chemokine receptor mutant mice which dominantly inhibited T_H1 *versus* T_H17 migration to the site of inflammation. T_H1 are thought to greatly rely on CCR5 and CXCR3 signalling for migration to peripheral sites. Mouse strains deficient in either of them develop EAE equal to or stronger than wt controls; interestingly, this is without showing a severe migratory deficit of T_H1 cells to the CNS. IFNγ levels, however, were drastically decreased in the mutant mice [TRAN EH ET AL. 2000, LIU L ET AL. 2006]. To prevent T_H17 migration, CCR6 seemed to be an ideal target since its great potential to direct specifically T_H17 cells to the CNS has been shown in different studies. Accordingly, a number of groups investigated on the effect of loss of CCR6 on the development of EAE. In the face of the simplicity of the question asked, biology proved to be extremely complex. Two of five studies reported milder EAE in $CCR6^{null}$ mutant mice [REBOLDI A ET AL. 2009, LISTON A ET AL. 2009], the other three studies described more severe disease development [YAMAZAKI T ET AL. 2008, VILLARES R ET AL. 2009, ELHOFY A ET AL. 2009]. The essence of all these experiments was that the integration of the chemokine receptor signalling in the whole immune response was too complex to allow clear conclusions on the question whether T_H1 or Th17 cells are crucial in EAE. CCR6 was found to be involved already in priming of T_H cells. It was also shown that T_{reg} express CCR6 and migrate towards CCL20 as T_H17 cells do and possibly interfere with EAE development. Taken together, the chemokine receptor mutant models did illustrate in their detailed analysis an influence of both, the T_H1 and T_H17 cells, in disease development but the necessity and sufficiency of the lineages was not convincingly shown. The most direct approach pursued to prove the role of the two effector types was passive induction of purified T_H1 or T_H17 cells. This experimental setup inherently contains a number of technical drawbacks. It is virtually impossible to purify an effector type to a hundred percent. Furthermore the model is rather artificial and error prone mainly due to the *in vitro* culture of the effector cells. Nevertheless, it is also fast and technically simple and contains a great deal of persuasive power. It was again LANGRISH ET AL. who early on presented most convincing data conveying the plain message that only T_H17 cells are encephalitogenic [LANGRISH CL ET AL. 2005]. That was somewhat surprising since adoptive transfer of T_H1 cells for passive induction of EAE had been a standard procedure for years. It needed three years for this issue to become resolved mostly due to the efforts of three studies in 2008 which have shown that in the autoimmune models EAE and EAU T_H1 cells in the absence of T_H17 cells can induce disease and are thus highly encephalitogenic [KROENKE MA ET AL. 2008, LUGER D ET AL. 2008, O'CONNOR RA ET AL. 2008]. Although differing in their perspective on the topic all three studies agree on that T_H1 and T_H17 committed T cells are at least redundant in their capacity to produce a clinically similar disease with comparable severity. This notion of redundancy is corroborated by the lack of a detrimental impact in terms of amelioration of disease upon deletion of signature cytokines like IFNγ, IL-12 and -18 as well as IL-17A, -17F, -21 and -22 [FERBER IA ET AL. 1996, WILLENBORG DO ET AL. 1996,

BECHER B ET AL. 2003, GUTCHER I ET AL. 2006, NINCHEN ET AL. 2007, SONDEREGGER I ET AL. 2008, COQUET JM ET AL. 2008, HAAK S ET AL. 2009].

The study on the development of EAU by CASPI and colleagues showed that upon adoptive transfer of T_H1 or T_H17 skewed T_H cells either set of effector cells can initiate disease [LUGER D ET AL. 2008]. At the same time KROENKE ET AL. reported a similar finding in the EAE model [KROENKE MA ET AL. 2008]. In the third study performed by the lab of ANDERTON, the authors put particular effort into delineating the purity and *in vivo* stability of their *in vitro* generated T_H1 and T_H17 cells [O'CONNOR RA ET AL. 2008]. Their T_H1 cells generated by stimulation with IFNγ and IL-18 infiltrated the brain in high numbers after adoptive transfer and did not show any capacity for IL-17A secretion when retrieved from spleen or CNS of diseased mice. In contrast, T_H17 cells (completely lacking IFNγ production) generated with TGFβ/IL-6/IL-23 in their hands generally failed to induce EAE. In a few cases, however, transfer of these cells did result in a very mild form of EAE with delayed onset. Lymphocytes isolated from the CNS of those animals always showed a marked $CD4^+$ T_H cell population producing IFNγ which might reveal some impurity or instability in the T_H17 lineage generated under the *in vitro* conditions. It seems to be a common feature that when *in vitro* polarised T_H17 cells are adoptively transferred in an EAE model T_H cells retrieved from the CNS of the diseased mice reveal a small population of $IFN\gamma^+$ cells. In spite of the many different approaches pursued, it is so far not clear whether the success of transfer of T_H17 cells rests on the small moiety of IFNγ producing T_H cells that can be subsequently found in the lesioned brain. Some of these cells are IL-17A/IFNγ double producers which elude clear categorization into the T_H1/T_H17 scheme (cf. "Discussion: T_H17 identity"). In apparent conflict with the notion that IFNγ is dispensable in CNS autoimmune inflammation, which is suggested by the strong EAE development in the IFNγ and IFNγR deficient mice, LUGER ET AL. found that in an adjuvant-free EAU model, IFNγ was actually crucially involved in the induction of disease [LUGER D ET AL. 2008].

Altogether it is intriguing that the engagement of two discrete effector arms of the adaptive immune system can lead to a hardly distinguishable clinical outcome, even though each arm induces a very different and distinct cascade of events. Owing to their differential chemokine profile, T_H1 or T_H17 biased local inflammation of the CNS correlates with a distinctly different cellular infiltration [CARLSON T ET AL. 2008]. While IL-23-driven T_H17 cells mostly induce expression of CXC chemokines (α-chemokines) bearing the ELR amino acid motif, T cells stimulated under the influence of IL-12 dominantly induce the expression of ELR^- CXC and CC (β-chemokines) chemokines. Accordingly, with regards to the composition of infiltrating leukocytes within the lesioned CNS an increase of the neutrophil fraction under T_H17 conditions and an increase of monocyte-like cells under T_H1 conditions has been shown [KROENKE MA ET AL. 2008]. There is comparable data suggesting the crucial importance of either leukocyte in EAE. One major T_H17 induced pathway of neutrophil recruitment to sites of inflammation is via CXCR2. Loss-of-function of this receptor leads to

a strong decrease of neutophilia in inflammatory models and a block of EAE in SJL and Balb/c mice. This was interpreted to mechanistically explain the supposed superiority of T_H17 cells in the induction of EAE [TAKAOKA A ET AL. 2001, CARLSON T ET AL. 2008]. As was mentioned before, IL-17A/IL-17F are the driving forces of T_H17 induced neutrophilia. Neutrophil mobilisation, however, is induced by a number of redundant pathways that are connected to all levels of immunity [LIU Y ET AL. 2009]. Nevertheless, there is evidence for intensive cross-talk between T_H17 cells and neutrophils that might explain their particularly strong connection. While it is established that T_H cells can directly influence neutrophils via GM-CSF, IFNγ and TNFα but only recruit them indirectly by the stimulation of stroma cell (cf. "Introduction: IL-23 induced effector cytokines"), it has recently been shown in the human system that neutrophils can talk back: Activated neutrophils can secrete CCL2 and CCL20, which are the chemoattractants sensed by CCR2 and CCR6 typically expressed on the surface of T_H17 cells [PELLETIER M ET AL. 2010]. CCL20 is not only a chemokine but also an antimicrobial factor, hence secretion might not only serve the purpose of T_H17 recruitment. More T_H17 cells at the site of inflammation result in more IL-17A/IL-17F which further recruits neutrophils. A recent study suggests that also neutrophils amplify their own recruitment by direct secretion of IL-17A as well as IFNγ in an acute inflammation model [LI L ET AL. 2010]. Once neutrophils accumulate at the site of inflammation, they quickly die by apoptosis which is a trigger for down regulating neutrophil recruitment. It was shown that phagocytosis of apoptotic neutrophils by APC curbs their capacity of IL-23 secretion which would inhibit the IL-23/T_H17/IL-17 axis [STARK MA ET AL. 2005]. While it is clear that T_H cells recruit neutrophils it remains unknown whether they also influence the quality of the neutrophil response according to the dominating T_H effector type. As compared to the strong connection between T_H17 cells and neutrophils, T_H1 cells preferentially mediate recruitment of monocytes/macrophages via the MCP-1/CCR2 axis as well as regulate macrophage activity. It is known from early works that this monocyte/macrophage activity also plays a crucial role in EAE development [TRAN EH ET AL. 1998, IZIKSON L ET AL. 2000, HUANG DR ET AL. 2001]. Interestingly, the dependence of EAE on the MCP-1/CCR2 axis seemed to be less pronounces is SJL mice as compared to C57BL/6 mice. This proposed qualitative difference in inflammation between the various T_H effector responses could account for the phenotypic differences in clinical signs of MBP induced EAE observed in mice lacking functional IFNγ signalling in the lab of OWENS [TRAN EH ET AL. 2000].

Besides the cellular composition of inflammatory infiltrates, their spatial distribution can also be directed via the degree of T_H1 or T_H17 dominance. It was first reported by the group of LAFAILLE that loss of IFNγ (T_H1 domination) caused a change in the clinical outcome of EAE from predominantly spinal cord inflammation to cerebellum/brain stem inflammation [WENSKY AK ET AL. 2005, LEES JR ET AL. 2008, STROMNES IM ET AL. 2008]. In both forms, T_H1 and T_H17 cells participate but either dominance shapes the spatial distribution of CNS inflammation differently. Within the brain, T_H17 cells were suggested to facilitate deep infiltration into the neuropil while the T_H1-driven infiltrate was found to be mainly

restricted to meninges, ventricles and vessels. One possible means of achieving parenchymal ingress of leukocytes in a TH17 associated inflammatory setting is via the pronounced induction of MMP, which play part in conferring passage over the *glia limitans* [reviewed in SHEN F ET AL. 2008, GOVERMAN J 2009]. This overall model would explain the divergent clinical and molecular phenotypes observed in EAE between the uses of different CNS derived peptides for immunisation or different strains of mice. Which TH effector type dominates is at least in part decided by the resulting avidity of the TCR:pMHC interaction at the initial priming and the local restimulation event resulting in a corresponding EAE phenotype. This model also reflects very well our findings on the role of TH17 effector cytokines in EAE.

Taken together, it is apparent that TH1 and TH17 cells are both present and both active at the inflammatory lesion. Both cell types contain encephalitogenic properties even though their individual modes of action seem to be largely uncoupled. Accordingly, loss of functional aspects of TH17 cells do influence the clinical outcome to some degree but do not render mice resistant to EAE. Instead, both effector types introduce a distinct licensing package into the adaptive immune response. The differential action of TH1 and TH17 cells might direct the spatial aspect of leukocyte infiltration as well as the recruitment of certain innate players in the inflammatory response which in concert could account for a different quality of long-term and short-term damage possibly explaining the more pronounced recovery in the IL-17A deficient mouse model.

Yet another TH effector cytokine, GM-CSF

This conclusion leads back to the original observation which implicated TH17 cells with autoimmunity; IL-23p19 deficient mice were found to be resistant to EAE while IL-12p35 deficient mice remained fully susceptible. Considering the data on the role of the known TH17 effector cytokines in CNS autoimmune inflammation collected to date, it is unlikely that this can be explained by loss of their effector potential. It is possible that TH17 cells harbour more undiscovered functional aspects that mediate their strong encephalitogenic characteristic. In our initial gene array screen for IL-23 induced, EAE associated genes upregulated by TH cells, GM-CSF was found to be upregulated alongside IL-17A, -17F and -22. Since the first description of TH17 cells, GM-CSF is known to be associated. Long before TH17 cells were formally designated as a distinct lineage, loss-of-function as well as gain-of-function studies have revealed a critical dependence of EAE development on GM-CSF production [MCQUALTER JL ET AL. 2001, MARUSIC S ET AL. 2002]. One aspect that was shown to be relevant in the context of EAE was the GM-CSF induced mobilisation and activation of monocytes which could potentially infiltrate the CNS perivascular space and differentiate into APC which could be involved in granting sufficient restimulatory potential to myelin reactive TH cells [GRETER M ET AL. 2005, KING IL ET AL. 2009]. Despite these promising results,

GM-CSF has not gained much scientific attention and was quickly outcompeted by IL-17A which was advertised in the paper by LANGRISH ET AL. to be essential in EAE. Our lab is currently following the lead on GM-CSF to elucidate its role in EAE [CODARRI L ET AL. 2010 (submitted)].

What does IL-23 really do?

In our study we also investigated on the impact of loss of IL-23 signalling on T_H cell behaviour under inflammatory conditions as compared to and in direct competition to wt T_H cells [GYÜLVÉSZI G ET AL. 2009]. To be able to pinpoint the functional defect that is conveyed by lack of IL-23 signals, we reconstituted RAG1^{null} mutant mice with a mix of either IL-12Rβ1^{null} (shared between IL-12 and -23) or IL-12Rβ2^{null} (IL-12 only) mutant together with wt bone marrow. The most obvious defect in the mice lacking IL-23 signalling is the inability of primed T_H cells to differentiate into effective T_H17 cells that could participate in EAE development. The surprise came with the finding that the additional lack of IL-23 signalling rendered the functional IL-12R-defective cells incapable to leave the SLT. This lack of CNS migration due to lack of IL-23R signalling is in accordance with a similar study by the group of CUA that was published shortly before our study [MCGEACHY MJ ET AL. 2009]. A closer look at the kinetics of the deficit in the IL-23R^{null} mutant T_H cells revealed that IL-23 signalling is required as early as four to six days *post* immunisation. This *in vivo* quality of IL-23 signalling in T_H17 differentiation was not revealed by the *in vitro* generation of T_H17 cells. While MCGEACHY ET AL. observed an impairment of the expansion of nascent T_H17 cells our results do not indicate this although we have not specifically addressed the degree of clonal expansion. Our as well as CUA'S study provide similar evidence that IL-23 is also required at the effector stage in the CNS. This observation, however, is harder to interpret because the few "T_H17" cells that do enter the CNS carry the early developmental deficit and thus do not necessarily represent activated T_H cells at the effector stage. For a simple DTH response as well as for actively induced EAE it has been shown that local application of IL-23 to IL-23p19^{null} T_H cells at the target organ recovers an effective T_H17 response [CUA D ET AL. 2003, MCGEACHY MJ ET AL. 2009]. At least two different scenarios could explain these findings: The early developmental deficit of the nascent T_H17 cell which interferes with their transmigration through the blood can be rectified by the exogenous, peripheral IL-23. In that case the assumption of the developmental arrest to occur after priming as well as its independence on the SLT microenvironment would be strengthened. It would, however, be also possible that the early phenotype in the nascent T_H17 cells is not essential for development but only effects cell mobility making CNS infiltration less likely but not impossible. In that case the T_H17 cells reaching the CNS would be true effector cells that require IL-23 to operate their pathogenic immune phenotype. The principle lack of any cytokine production of IL-12Rβ1^{null} T_H cells observed in our study rather suggests that those cells do carry a general developmental deficit. In respect to the migration phenotype our

results support evidence generated in the lab of EGWUAGU in 2008. Their study examines the complete resistance of ($CD4^{cre}$ x $STAT3^{flx/flx}$) mutant mice in EAE and EAU. In the latter model the authors correlate the lack of primed retina-antigen specific TH cell ingress into the eyes with a substantial decrease of cells expressing α4-, β1-integrin chains [LIU X ET AL. 2008]. The α4-integrin chain dimerises with either the β1-chain or the β7-chain. The α4β1-integrin is also known as very late antigen 4 (CD49d–CD29) and the α4β7-integrin is referred to as *lamina propria* associated molecule 1. The α4β1-integrin:VCAM-1 interaction is crucially involved in *trans*-endothelial migration of leukocytes and recruitment of T cells into the retina. The deficit of adhesion molecule regulation could at least in part explain the phenomenon of lack of TH cell migration when IL-23 signalling is defective.

While IL-23/STAT3 signalling is clearly important *in vivo*, the *de novo* generation of TH17 cells *in vitro* does not require IL-23 signalling. This is one of the obvious shortcomings inherited to all *in vitro* based experimental TH17 settings fuelling concern about the informative value of those results. Nonetheless, there are interesting observations based on the *in vitro* generation of TH17 cells. In short-term cultures IL-23 was suggested to be involved in expansion and survival of TH17 cells [VELDHOEN M ET AL. 2006]. Correspondingly, it was found to be required for lasting IL-17A production [CHEN Z ET AL. 2006]. In a more elaborate studies on long-term cultures of TH17 cells IL-23 was shown to maintain TH17 characteristics of the cells but not to be sufficient to generate lineage commitment [STRITESKY GL ET AL. 2008]. A recent study by the lab of WEAVER contradicts to some extend highlighting continuous TGFβ signalling to be essential for long-term TH17 stability [LEE YK ET AL. 2009]. Earlier findings from CUA'S lab, however, indicated that TGFβ signalling to "mature" TH17 cells induces a phenotypic shift to an IL-10 producing TH17 cell which is indicative for a more regulatory effector function [MCGEACHY MJ ET AL. 2007]. Concerning the potential role of IL-23 for the effector phase of TH17 cells it has been found that IL-23 can substantially shape the cytokine response of TH17 cells, e.g. by the induction of IL-22 production [MCGEACHY MJ ET AL. 2007, LIU X ET AL. 2008, LEE YK ET AL. 2009]. This and other finding revealed an unexpected degree of heterogeneity of the TH17 lineage which will be discussed at a later point (cf. "Discussion: TH17 identity").

The receptor for IL-23 is also expressed by other haematopoietic cells [AWASTHI A ET AL. 2009], including dendritic cells and macrophages, and it is becoming increasingly clear that the functions of IL-23 extend beyond TH17 cell biology. In a T cell independent model of IBD the group of POWRIE has found a protective role of IL-23 [UHLIG HH ET AL. 206]. In infection IL-23 deficiency abrogates host protection against *Citrobacter rodentium*, even in the presence of normal populations of TH17 cells as the frequency of $IL\text{-}17A^{+}$ T helper cells in the intestine at steady state or after infection was not affected by the absence of IL-23 [MANGAN PR ET AL. 2006, ZHENG Y ET AL. 2008]. Concerning autoimmune neuroinflammation it remains to be clarified which impact IL-23 signalling to innate lymphoid and myeloid cells truely has. It only became visible in the last two years that along with any TH17 driven inflammatory

response a considerable amount of the TH17 cytokines produced are in fact produced by innate scource. Theirs and other IL-23 responsive cell's contribution in autoimmune neuroinflammation remains to be clarified [SUTTON CE ET AL. 2009].

Mice lacking IL-23 are completely resistant to EAE induction, but so far there was no clear indication of the role of IL-23 in humans. A recent clinical study by SEGAL and colleagues using p40-blocking antibody (p40), subunit shared by IL-12 and -23) in relapsing–remitting MS (RRMS) patients showed an unexpected result [SEGAL BM ET AL. 2008]. In their study, the neutralisation of p40 does not significantly affect the course of MS and the formation of white matter lesions. This observation raises several questions. Apart from possible technical issues the possibility remains that the function of IL-23 in MS varies between mice and humans or that the applied animal models do not depict this aspect of the human disease accurately. However, IL-23 may also act differently in certain forms of MS such as RRMS and chronic progressive MS, as the clinical trial only included RRMS patients. It is essential to learn more about the role of IL-23 in an ongoing effector response in mice and humans to enable proper targeting of clinical intervention which might then result in more promising results or else reveal the nature of that mechanistic difference between human MS and murine EAE.

TH17 identity

The discovery and continuous characterisation of the diversity of proinflammatory TH subset has greatly broadened our view on the adaptive immune response in health and disease. It is undisputable that there is still a lot to learn about their differentiation, their mode of action and their position in the intact immune system. The current progress in the understanding of the role of TH cells in CNS autoimmunity already indicates that even a fairly simple disease model such as EAE cannot truly be attributed to the dominant action of a single TH cell line. TH1 and TH17 cells are both present and both active at the inflammatory lesion. The data presented in this study made a critical contribution to the claim of a revision of the role of IL-23 induced effector cytokines in organ specific autoimmunity. This revision is currently followed by a revision of the identity of TH17 cells themselves.

Among other aspects, a lineage is defined by the incorporation of exclusive effector mechanisms, like specific signature cytokines. While there is little overlap between TH1 and TH2 cells in that respect [HEGAZY AN ET AL. 2010], the TH17 effector type has opened an extensive "grey-zone". IL-17A/IFNγ, IL-17A/IL-4, IL-17F/IFNγ and IL-22/IFNγ double producers have been reported of which IL-17A/IFNγ double positive cells seem to be most frequent and are by now established as a common *in vivo* phenomenon under inflammatory conditions [ALBANESI C ET AL. 2000, LUGER D ET AL. 2008]. Correspondingly it was found that T-bet and RORγt are active in these double positive clones [ANNUNZIATO F ET AL. 2007]. It seems that there is hardly any adoptive transfer setting where TH17 are fully stable *in vivo*

which imposes the notion that TH17 cells are plastic throughout their ontogeny. Early studies performed by the lab of KAPLAN already pointed out that TH17 cells do not fulfil the requirement of terminal lineage commitment because of continuous instability which was governed by the intrinsic bias to convert into the TH1 phenotype [MATHUR AN ET AL. 2007, STRITESKY GL ET AL. 2008]. Probably the most striking *in vivo* observation illustrating the relevance of this finding was reported in a mouse model of type I diabetes. Efficient disease development was induced by transfer of TH17 cells. However, this disease induction could only be prevented by the application of IFNγ, but not of IL-17A neutralising antibodies. It was found by two groups independently that highly purified TH17 cell, devoid of any IFNγ secretion, readily converted into TH1-like cells *in vitro* and *in vivo*, which in the case of the NOD diabetes model carry all the pathogenic potential [BENDING D ET AL. 2009, MARTIN-OROZCO N ET AL. 2009]. Similar observations in terms of lineage instability were made in EAE, EAU and colitis.

Considering the strong link between TH1 and TH17 cells and cytokines, it appears far fetched to envision those cells as antagonistic and exclusive lineages. There is considerable evidence for a more complex interconnection [KRYCZEK I ET AL. 2008A & 2008B, YANG Y ET AL. 2009], which reinstate the former idea of TH1/TH17 synergism [KAWAGUCHI M ET AL. 2001]. Also in respect of classical TH2 dominated pathophysiologic conditions, TH17 activity can be synergistic. Studies on acute airway hypersensitivity have revealed a significant contribution of TH17 effector cytokines on neutrophil and eosinophil recruitment to the lung [YANG XO ET AL. 2008, HAAK ET AL. (unpublished data)].

TH17 cells exhibit conspicuous differences in the modality of lineage development compared to TH1 and TH2 cells. On the one hand, TH17 cytokines lack prominent suppressive capacity on the competing lineages, on the other, TH17 cells themselves remain highly dependent on the absence of those TH1/TH2 cytokines as well as the presence of TH17 differentiating factors. A single study reports the capacity of IL-17A to inhibit TH1 differentiation. The general relevance of this finding has to be proven by further studies.

Epigenetic screens have confirmed the programmatic instability of the TH17 effector type, even though this level of transcriptional regulation just started to be experimentally explored. In line with the strongly polarised phenotypes the effector cytokines of TH1, TH2 and TH17 cells are reciprocally counter-regulated by histone modifications which mark gene loci as permissive or silenced accordingly. The permissive acetylation of H3 at the IL-17A/IL-17F promoter region in TH17 cells has been shown to be STAT3 mediated. The subtle difference between the T cell types lies in the accessibility of the gene loci of the respective TF. All "master TF" of lineage commitment remain accessible in all lineages with the exception of *rorc* which is inactivated in all lineages but TH17 and T_{reg} [AVNI O ET AL. 2002, AKIMZHANOV AM ET AL. 2007, SCHOENBORN JR ET AL. 2007, WEI G ET AL. 2009]. nT_{reg} and TGFβ induced iT_{reg} might differ a bit in their status of the *il17a* and *rorc* loci. In both cell types *il17a* was found to be inactivated reflecting the inhibitory effect of FoxP3. *Rorc*,

however, was activated in iT_{reg} while marked bivalent in nT_{reg}. This finding is not unexpected since TGFβ induces *rorc* transcription [ZHOU L ET AL. 2008, WEI G ET AL. 2009].

While the genomic analysis of the different T_H effector types in most parts complement the experiences on the cellular level it has not yet revealed an epigenetic clue for the apparent T_H1 bias in the instability of the T_H17 phenotype. Even disregarding the fact of T_H17 instability, also in the "mature" phenotypic state an intimate link between T_H1 and T_H17 cells is reflected on different molecular levels. Among the effector cytokines they produce there seems to be an overlap unrepresented by any other pair of T_H effector types. TNFα for one is long known to be secreted by both lineages. A highly ambitious multiparametric computational analysis on human T_H subsets presented evidence of another common factor [VOLPE E ET AL. 2009A & 2009B]. In a pairwise correlation analysis IL-22 expression was found to correlate better with IFNγ than with IL-17A expression. Similar were the results for the cytokine-TF correlation. While IL-17A and IL-17F were confirmed to be highly connected to RORγt, RORα and also to AHR, IL-22 was connected with T-bet expression. In mice, in contrast, independent studies suggest a strong link between IL-22 and AHR which brings up the question whether the transcriptional regulation of IL-22 might be partially different in mice and humans. The concept of a transcriptional connection between IL-22 and IFNγ is compelling as they are encoded in close proximity within the same gene cluster and common regulatory elements would be possible. In mice and humans this locus is considerably different as complex segmental duplications doubled their interspace in mice (result in loss of functional IL-26), which could potentially alter transcriptional regulation. Interestingly, T cell blasts from patients with defective IL-12 signalling have been reported to secrete less IL-22 [DE BEAUCOUDREY L ET AL. 2008]. Further similarities of IL-22 and IFNγ are given in their structure, the receptor family they use and partially their functional context (e.g. tissue repair).

Coming back to the comparison between T_H1 and T_H17 cells, also on the cytokine receptor level interesting links have been observed. The receptor of the T_H1 cytokine IL-18 (IL-18R) is also expressed on T_H17 cell. IL-18 can be used *in vitro* to potentiate the effect of IL-23 on T_H17 cells [MATHUR AN ET AL. 2006, WEAVER CT ET AL. 2007]. Only recently it was discovered that T_H17 even after extensive polarisation do not lose IL-12Rβ2 expression on their cell surface [LEE YK ET AL. 2009]. IL-12 signalling is a hallmark feature of T_H1 differentiation as it upregulates STAT4. IL-12/STAT4 can amplify the IFNγ response of T_H1 cells. Nevertheless, IL-23, while strongly relying on STAT3 activation, also signals via STAT4. STAT4 is necessary for IL-23 driven IL-17A expression and has been shown to mediate functional aspects of T_H17 effector response *in vivo* [OPPMANN BR ET AL. 2000, MATHUR AN ET AL 2007, FURUTA S ET AL. 2008, reviewed in KAPLAN MH 2005]. This marks STAT4 dependence as another shared feature specifically between the T_H1 and T_H17 phenotypes.

The second T_H1 driving TF is T-bet. T-bet as well as STAT4 deficient mice have a severe defect in T_H1 cell differentiation; however, only double mutant mice lack this lineage

completely [FURUTA S ET AL. 2008]. While it is widely accepted that T-bet expression in TH cell is exclusive to the TH1 effector type, T-bet expression in nascent TH17 cells has become an interesting concept that completely challenges the current view on TH cell identities. The expression of T-bet has long been correlated with TH1 and TH17 driven autoimmune diseases [BETTELLI E ET AL. 2004, LOVETT-RACKE AE ET AL. 2004, NATH N ET AL. 2006, GOCKE AR ET AL. 2007]. The group of LOVETT-RACKE presented compelling evidence that suggest that T-bet is essential for the encephalitogenic potential of TH17 cells. From the work of MCGEACHY ET AL. and XU ET AL. it is known that not all IL-17$^+$ TH cells are encephalitogenic. TGFβ, which in combination with STAT3 signalling is a warrantor of high yields of *de novo* TH17 differentiation, has been shown to induce an anti-inflammatory bias in mature TH17 cell. There is some evidence in the human system that TGFβ promotes TH17 differentiation via downregulation of T-bet [SANTARLASCI V ET AL. 2009]. On the other hand, IL-23 and possibly other STAT3 signalling cytokines drive or sustain an encephalitogenic phenotype [MCGEACHY MJ ET AL. 2007, XU J ET AL. 2009]. Interestingly, the LOVETT-RACKE lab presented evidence that T-bet expression in TH17 cells upregulates the IL-23R, which provides a potential mechanistic link between the observed T-bet expression in TH17 cells and its correlation with encephalitogenicity [GOCKE AR ET AL. 2007, YANG Y ET AL. 2009].

Taken together, the current status of the ongoing analysis of late TH effector lineage plasticity has revealed a so far unacknowledged level of plasticity within the whole field of TH differentiation. While the genetic space can be drastically confined upon continuous polarising input by epigenetic modifications, the studies conducted so far as well as the experiences on the cellular level addressing cell fate commitment corroborate the notion that even in a most terminally differentiated state there are still major genetic triggers transcriptionally accessible that could potentially redirect lineage commitment and reinstall some degree of pluripotency. How physiologically relevant these loopholes in cell fate commitment are *in vivo* still has to be explored. But nonetheless, the knowledge of the major molecular pathways and detailed mapping of epigenetic changes during effector lineage differentiation is of paramount value to medical and basic science, as it will reveal the means and boundaries of interventions in the manipulation of cell identities as well as the impact of genetic mutations that predispos for disease susceptibility in the human system. The most impressive illustration of the power of this approach is the recent achievement of redirecting terminally differentiated fibroblasts towards pluripotent stem cells with help of as few as four transcription factors (c-Myc, SOX2, Klf4, Oct4) [WOLTJEN K ET AL. 2009].

Most dramatic, however, are the implications of recent findings on the perception of the TH17 effector type. While our study contributed in the revision of the known TH17 effector mechanisms in respect to their role in autoimmunity, others, at the same time, provided clear evidence that TH17 cells themselves are also not essential for disease development. Under physiologic conditions their individual proinflammatory potential, although

employing distinct aspects of the immune response, most likely act in concert while revealing an intimate phenotypic and developmental relationship. While the T_H17 phenotype is active during the acute phases of inflammation this effector type seems short lived and to convert according to external stimuli but preferentially into T_H1 effector cells. It can be expected that the coming years will lead to a reorganisation of the perception on the structure of a T cell driven immune response. For one, after a short term of consensus on the distinct lineage identity of T_H17 cells, it has become evident that this effector type does not seem to exist as an independent lineage.

A number of different speculative scenarios could be envisioned of how the T_H17 effector type might has developed. At the gut mucosa the constant interaction with comensal microorganisms demands a high degree of flexibility of the immune system. At this interface T_H17 and T_{reg} effector cells are making the scene and also $FoxP3^+$ $ROR\gamma t^+$ T cells have been described. The microflora keeps the immune system activated but tolerised, which is a necessary equilibrium since friend an foe are identical; micro-organisms of the gut-flora can turn into life threatening pathogens by mere changes in the composition of the colonising strains. The plasticity between T_{reg} and T_H17 might be the foundation of this context dependent flexibility in tolerance *versus* rejection [AYYOUB M ET AL. 2009]. Looking at the whole body's defence, T_H17 cells have been most specifically connected to clearance of infections of extracellular bacteria and fungi. Many of these pathogens are highly organised and difficult to contain (host cell independent, mobile etc.). The T_H17 response might represent the most aggressive pole of a cell mediated inflammatory immune response that allows deep tissue penetration and massive recruitment of granulocytes. Neutrophils, along with the IL-17A/IL-22 activated stroma, secrete a great variety of antimicrobial substances that can kill extracellular micro-organisms. It is conceivable that such a polarised immune response can not be meant to be permanently active. Accordingly, the intrinsic program of T_H17 cells precludes extensive tissue damage by its programmatic phenotype instability, which mostly results in a conversion into a T_H1-like phenotype. The T_H1 effector repertoire includes macrophage- and possibly IL-22-mediated tissue repair and late phase immune responses.

Summary

Experimental autoimmune encephalomyelitis (EAE) serves as the animal model of multiple sclerosis, which is an inflammatory disease of the central nervous system and thought to be mediated by an autoimmune process. EAE is mainly driven by T helper (T_H) cells, which can be divided into several subtypes with distinct phenotypes and functions. IL-12 induced T_H1 cells were long believed to be the essential disease promoting cell population, until the discovery that IL-23, and not IL-12, is crucial for EAE development. This initiated a paradigm shift and led to the notion that IL-23 driven, IL-17A secreting T_H17 cells are actually mediating the disease. Most data supporting this perception stems from correlative studies associating the mere presence of IL-17A or T_H17 cells at the site of inflammation with an autoimmune pathogenic function. However, we could show that in contrast to IL-23, IL-17A is not essential for the development of EAE. Hence, we understand that other IL-23 induced factors and/or mechanisms exist mediating encephalitogenicity, the revealing of which's identity this study was designed for.

We identified IL-17F as well as IL-22 to be induced by IL-23 in disease associated T_H17 cells and generated IL-17F^{null} and IL-22^{null} mice to specifically determine their role in autoimmune inflammation. We found IL-17F^{null} as well as IL-22^{null} mice to be fully susceptible to EAE contrasting the notion that genes associated with T_H cells present at the site of inflammation imply their pathogenic function. To exclude the possibility of a compensatory effect between IL-17A and IL-17F we induced disease in IL-17Fnull mice treated with an neutralising anti-IL-17A antibody. Also those mice were fully suceptible to EAE showing that none of the so far identified IL-23 induced factors mediate encephalitogenicity. To further investigate the mechanistic impact of IL-23 on T_H cells and their behaviour, we generated bone marrow-chimeric mice in which we could trace individual populations of IL-23 responsive or unresponsive T_H cells during EAE. Loss of IL-23 signalling eliminated the ability of T cells to invade the CNS. Therefore, IL-23 seems to license T cells to invade the CNS and to exert their effector functions at this site of inflammation.

Taken together, we show that self-reactive T_H cells coexpress IL-17A, IL-17F and IL-22, but that none of these cytokines are the mediators of encephalitogenicity while the essential role of IL-23 in EAE might at least in part be explained by its licensing of T cells to invade the CNS.

Zusammenfassung (German translation)

Experimentelle autoimmune Encephalomyelitis (EAE) ist das Tiermodell für Multiple Sklerose, eine entzündliche, sehr wahrscheinlich autoimmune Erkrankung des Zentralen Nervensystems. EAE wird hauptsächlich durch T Helfer (T_H) Zellen vermittelt, die aufgrund ihrer jeweils spezifischen Phänotypen und Funktionen in verschiedene Untergruppen unterteilt werden können. IL-12 induzierte T_H1 Zellen galten lange als wichtigste pathogene Zellpopulation, bis entdeckt wurde, dass IL-23 und nicht IL-12 für die Entwicklung von EAE essentiell ist. Diese Entdeckung führte zu einem Paradigmenwechsel und der Auffassung, dass IL-23 induzierte, IL-17A sekretierende T_H17 Zellen die Krankheit vermitteln. Der Grossteil der Daten, die diese Annahme stützen stammt allerdings aus korrelativen Studien, die die schlichte Anwesenheit von IL-17A oder T_H17 Zellen am Entzündungsherd mit einer pathogenen Funktion gleichsetzen. Allerdings konnten wir zeigen, dass IL-17A, im Gegensatz zu IL-23, keine Voraussetzung für die Entwicklung von einer autoimmunen Entzündung darstellt. Demzufolge gehen wir davon aus, dass andere IL-23 induzierte Faktoren und/oder Mechanismen existieren, die Enzephalitogenität vermitteln und zu deren Identifizierung wir diese Studie durchgeführt haben.

Wir konnten zeigen, dass IL-17F und IL-22 durch IL-23 induziert und von krankheitsassoziierten T_H17 Zellen exprimiert werden und haben zur gezielten Bestimmung ihrer Rolle in autoimmuner Entzündung IL-17F^{null} und IL-22^{null} Mäuse hergestellt.

IL-22^{null} und IL-17F^{null} Mäuse entwickelten EAE mit normalem Verlauf, was der Annahme, dass mit am Entzündungsort vorhandenen T Zellen assoziierte Gene eine pathogene Funktion bedeuten, widerspricht und zeigt, dass keiner der bisher identifizierten, IL-23 induzierten Faktoren ausschliesslich für die Entwicklung von EAE verantwortlich ist. Um nun den Mechanismus der Wirkung von IL-23 auf T_H Zellen und ihr Verhalten dieser Zellen genauer zu untersuchen, haben wir Knochenmarkschimären hergestellt, in denen wir individuelle Populationen von auf IL-23 reagierenden und nicht reagierenden T_H Zellen in EAE verfolgen konnten. Durch den Verlust von IL-23 Signalübertragung konnten die T Zellen nicht mehr zu dem ZNS migrieren. Demnach scheint IL-23 T Zellen zu lizensieren, das ZNS zu infiltrieren und dort ihre Effektorfunktionen ausführen zu können.

Zusammenfassend zeigen wir, dass selbstreaktive T_H Zellen IL-17A, IL-17F und IL-22 koexprimieren aber dass keines dieser Cytokine die Enzephalitogenität vermittelt. Hingegen könnte die essentielle Rolle von IL-23 in EAE unter anderem durch die ermöglichten Migration von T_H Zellen zum ZNS erklärt werden.

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Th17 cells in autoimmune disease: changing the verdict.

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IL-17A and IL-17F do not contribute vitally to autoimmune neuro-inflammation.

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IL-22 is expressed by Th17 cells in an IL-23-dependent fashion, but not required for the development of autoimmune encephalomyelitis.

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